A New Class of Molecules from *Staphylococcus* Species Affects Quorum Sensing and Growth of Gram-Negative Bacteria

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Abbreviations

2D	Two-dimensional
ACN	Acetonitrile
Agr	Accessory gene regulation
AHL	N-acyl homoserine lactone
AI-2	Autoinducer-2
AIP	Autoinducer peptide
ATCC	American Type Culture Collection
BA	Bovine serum
BSA	Bovine serum albumin
CFU	Colony forming unit
DMSO	Dimethyl sulfoxide
DSMZ	Deutsche sammlung von Mikroorganismen und Zellkulturen GmbH
DTT	Dithiothreitol
ESI-MS	Electrospray ionization mass spectrometry
EtOH	Ethanol
FT-ICR-MS	Fourier transform ion cyclotron resonance mass spectrometry
g	Gram
GC-EI-MS	Gas chromatography electrospray ionization mass spectrometry
GC-MS	Gas chromatography mass spectrometry
h	Hour
HAI-1	N-(3-hydroxybutyryl)-homoserine lactone
HCl	Hydrogen chloride
HPLC	High performance liquid chromatography
HSL	Homoserine lactone
HSQC	Heteronuclear single quantum Coherence
K_2HPO_4	Potassium hydrogen phosphate
KCl	potassium chloride
LB	Lysogenic broth
M	Molar (mol/l)
m	Milli
m/z	Mass-to-charge ratio
MB	Marine broth
MeOH	Methanol
$MgCl_2$	Magnesium chloride
MHz	Mega Hertz
min	Minute
ml	Milliliter
MLST	Multilocus sequence typing
MS	Mass spectrometry
NaCl	Sodium chloride
nm	Nanometer
NMR	Nuclear magnetic resonance
OD	Optical density

OEG	Oligo-ethylene-glycol
PBS	Phosphate-buffered saline
PEG	Poly-ethylene-glycol
Phe	Phenylalanine
QS	Quorum sensing
ROS	Reactive oxygen species
RP-HPLC	Reversed phase HPLC
rRNA	Ribosomal RNA
R_t	Retention time
Sar	Staphylococcus accessory regulation
SDS	Sodium dodecyl sulphate
SIG	*Staphylococcus intermedius* group
Trp	Tryptophan
TSB	Tryptic Soy Broth
Tyr	Tyrosine
UV	Ultraviolet
WT	Wild type
WTA	Wall teichoic acid
yayurea A	N-[2-(1H-indol-3-yl)ethyl]-urea
yayurea B	N-(2-phenethyl)-urea

Symbols

µ	Micro
^oC	Degree(s) Celsius
%	Percentage
γ	Gamma
Δ	Deletion

Summary

This thesis addressed bacterial communication between Gram positive and Gram negative bacteria. The knowledge that many pathogens rely on cell-to-cell communication mechanisms known as quorum sensing, opens a new disease control strategy: quorum quenching. While studying the interaction of staphylococci with Gram-negative bacteria, we came across another communication system in a *Staphylococcus intermedius* group (SIG) species which can act as a zoonotic pathogen. This is one of the rare examples where Gram-positive bacteria excrete two compounds in comparatively high amounts that suppress the quorum sensing signaling and inhibit the growth of a broad spectrum of Gram-negative beta- and gamma-proteobacteria. The excreted compounds, named yayurea A and B, were isolated from *S. delphini* and structurally characterized. *In vitro* studies with the N-acyl homoserine lactone (AHL) responding receptor LuxN of *V. harveyi* indicated that both compounds stimulated the LuxN-mediated phosphorylation of LuxU and caused the opposite effects with AHL. Furthermore, growth of yayurea A and B producing *S. delphini* is not suppressed by respiratory toxins when co-cultured with *P. aeruginosa.* Taken together, yayureas are presumably involved in self-protection and ensuring competitiveness in natural environments shared with Gram-negatives. In the second part of thesis, the role of protein A in *Staphylococcus aureus* adhesion to ethylene glycol coated surfaces was investigated. It was found that the coating materials, HS-$(CH_2)_{11}EG_3OMe$ (EG_3OMe) and poly-ethylene-glycol (PEG) prevented bacterial adhesion of *S. aureus* cells. However, pretreatment of EG_3OMe and PEG coatings with γ-globulins or serum strongly promoted adherence of *S. aureus* cell. Furthermore, the *spa*-deletion mutant *S. aureus*, lacking the IgG binding protein A, showed reduced adherence and pretreatment of *S. aureus* with serum significantly decreased adherence. These results suggested that γ-globulins play a crucial role in promoting *S. aureus* cells adhesion by its IgG binding proteins. Particularly γ-globulins bound to the coated surfaces thus mediating adherence of *S. aureus* via its protein A. Intercepting this adherence should prove to be useful in preventing biofilm formation.

Zusammenfassung

Diese Arbeit behandelt bakterielle Kommunikation zwischen Gram-positiven und Gram-negativen Bakterien. Mit dem Wissen, dass viele Krankheitserreger auf Zell-zu-Zell-Kommunikation setzen (Quorum Sensing), öffnet sich eine neue Strategie der Krankheitsbekämpfung: Quorum quenching. Während Interaktionsstudien von Staphylokokken mit Gram-negativen Bakterien sind wir auf ein neuartiges Kommunikationssystem gestoßen in dem zwei Substanzen in vergleichsweise hohen Mengen von Bakterien der *Staphylococcus intermedius* Gruppe ausgeschieden werden, welche Quorum Sensing-Signale und das Wachstum eines breiten Spektrums von Gram-negativen beta-und gamma-Proteobakterien unterdrücken. Die ausgeschiedenen Verbindungen, yayurea A und B, wurden von *S. delphini* isoliert und strukturell charakterisiert. *In vitro*-Studien mit dem N-Acylhomoserinlacton (AHL)-Rezeptor LuxN von *V. harveyi* zeigten, dass beide Verbindungen die LuxN-vermittelte Phosphorylierung von LuxU stimulieren und den entgegengesetzten Effekt zu AHL verursachen. Darüber hinaus wird das Wachstum des Yayurea A und B produzierenden *S. delphini* nicht durch die von *P. aeruginosa* produzierten Atemgifte während der Kokultivierung gehemmt. Zusammengefasst ist zu vermuten, dass Yayureas beim Selbstschutz und der Sicherung der Wettbewerbsfähigkeit der Produzenten in der natürlichen Umgebung mit Gram-negativen Bakterien beteiligt sind. Im zweiten Teil der Arbeit wurde die Rolle von Protein A bei der Adhäsion von *S. aureus* an Ethylenglykol beschichteten Oberflächen untersucht. Wir konnten zeigen, dass die Stoffe HS-(CH2) 11EG3Ome (EG3OMe) und Polyethylenglykol (PEG) die bakterielle Adhäsion von *S. aureus*-Zellen verhindern können. Die Vorbehandlung der EG3OMe und PEG Beschichtungen mit γ-Globulinen oder Serum führte jedoch zu einer starken Erhöhung der Adhäsion von *S. aureus* Zellen. Sowohl die *spa*-Deletionsmutante, als auch die Vorbehandlung von *S. aureus* mit Serum bewirken verminderte Adhäsion. Diese Ergebnisse legen nahe, dass die an die an die beschichteten Oberflächen gebundenen γ-Globuline die Adhäsion von *S. aureus* mittels Protein A vermitteln. Diese Adhäsion zu unterbinden könnte sich bei der Präventionen von Biofilmbildung als nützlich erweisen.

Part I Structure and Function of Yayureas from *Staphylococcus intermedius* group

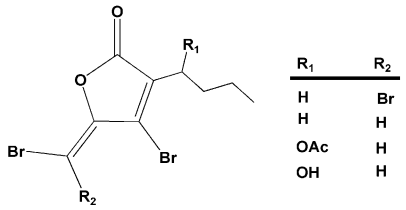
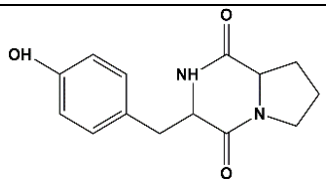
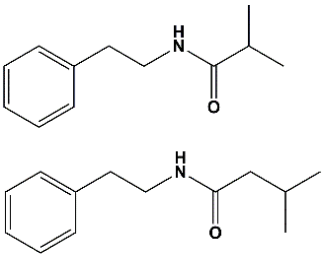
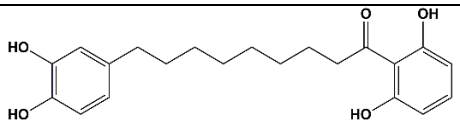
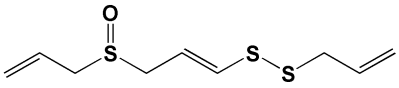
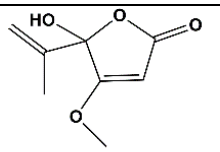
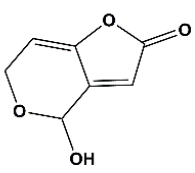
Introduction

1. Quorum sensing (QS)

Quorum sensing (QS) is a process of bacterial communication, first discovered in the marine photobacteria *Vibrio fischeri* (Nealson et al., 1970). The signal transduction of QS is mediated through the synthesis and secretion of small membrane-diffusible hormone-like molecules termed autoinducers. When they accumulate and reach a critical threshold, autoinducers bind to cognate receptors and trigger QS-regulated gene expression. The signal-activated receptor directly or indirectly controls the expression of target genes. Since the concentration of signaling molecules in liquid culture is proportional to cell density, gene expression is regulated in response to bacterial population densities (Atkinson and Williams, 2009; Waters and Bassler, 2005). QS empowers bacteria to coordinate pathogenicity, cell division (Swift et al., 1996), sporulation (Jabbari et al., 2011), conjugation (White and Winans, 2007), biofilm formation, swarming, toxin production (Falcao et al., 2004), bioluminescence (Miyamoto et al., 2000) and production of secondary metabolites (Swift et al., 1996) to rapidly respond to the environment.

There is growing interest in utilizing QS as a new prospective therapy against bacterial infection, as the evolutionary pressure to develop drug resistance with QS inhibitor treatment is less strong. In contrast to classic antibiotics, quorum-quenching compounds are inhibitors of bacterial virulence, rather than of bacterial growth (Cegelski et al., 2008), and therefore might serve as promising anti-virulence therapies (Atkinson and Williams, 2009; Njoroge and Sperandio, 2009). Following studies on halogenated furanones, well-known QS inhibitors from the red marine alga *Delisea pulchra* (Gram et al., 1996; Manefield et al., 2001; Rasmussen et al., 2000), screening for potential QS inhibitors has led to the discovery of many compounds with drug development potential **(Table 1)** (Gonzalez and Keshavan, 2006).

Table 1. Chemical structures of quorum sensing inhibitors

Names	Source	Structure	Active against
Furanones (Gram et al., 1996)	*Delisea pulchra*		*P. aeruginosa*
Cyclo-L-proline-L-tyrosine (Teasdale et al., 2011)	*Bacillus cereus* D28		*V. harveyi*
N-(2'-phenylethyl)-isobutyramide 3-methyl-N-(2'-phenylethyl)-butyramide (Teasdale et al., 2009)	*Halobacillus salinus C42*		*V. harveyi* and *C. violaceum*
Malabaricone C (Chong et al., 2011)	*Myristica cinnamomea*		*C. violaceum* and *P. aeruginosa*
Ajoene (Bjarnsholt et al., 2005)	*Garlic alliinase*		*P. aeruginosa* and *V. harveyi*
Penicillic acid Patulin (Rasmussen et al., 2005)	*Penicillium coprobium*	 	*P. aeruginosa*

2. QS systems of Gram-negative bacteria

The first QS circuit was discovered in *V. fischeri*, and was responsible for population density dependent bioluminescence. The autoinducer (3-oxo-C6-homoserine lactone), which is synthesized by LuxI, binds to the LuxR protein and activates the *lux* operon producing the bioluminescence. In *V. harveyi*, the QS system consists of three autoinducers and three cognate receptors functioning in parallel to channel information into a shared regulatory pathway (Waters and Bassler, 2005). Similar to *V. fischeri*, *V. harveyi* produces an AHL signal termed HAI-1 (3-hydroxy-C4-homoserine lactone) (Hanzelka et al., 1999) which binds to a membrane-bound sensor histidine kinase (LuxN). The second autoinducer molecule is AI-2, a furanosyl borate diester, which binds to the periplasmic protein LuxP. The LuxP-AI-2 complex then interacts with another membrane-bound sensor histidine kinase known as LuxQ. The third autoinducer molecule is termed CAI-1 (cholera autoinducer-1), a long chain ketone (Chen et al., 2002; Higgins et al., 2007), which is recognized by the membrane-bound sensor histidine kinase CqsS (Henke and Bassler, 2004). At low cell density, and in the absence of appreciable levels of autoinducers, the three sensors (LuxN, LuxQ, and CqsS) act as autophosphorylating kinases that subsequently transfer the phosphate to the cytoplasmic protein LuxU, which then passes the phosphate to the DNA-binding response regulator protein LuxO (Freeman and Bassler, 1999a, b). Phosphorylated LuxO represses the master regulator of QS, LuxR, via the sigma factor σ^{54} and small regulatory RNAs (Lenz et al., 2004; Lilley and Bassler, 2000).

P. aeruginosa is a common bacterium that causes diseases in humans and other animals. It is viable on and in medical equipment, causing cross-infections in hospitals, and is responsible for about one in ten hospital-acquired infections by *Pseudomonas* species. Pathogenicity is caused by several QS-controlled factors, such as exoenzymes, exotoxins, virulence factors, and biofilm formation. For example, the *P. aeruginosa* exotoxin A inactivates elongation factor 2 of eukaryotic cells, leading to translation inhibition. Biofilms protect *P. aeruginosa* from antibiotic treatment and enable chronic opportunistic infections. Similar to *V. harveyi*, *P. aeruginosa* coordinates the expression of nearly 10% of its genome through three hierarchically arranged QS systems, namely Las, Rhl and Pqs (Williams and Camara, 2009). Each system consists of enzymes involved in autoinducer synthesis

and the target regulator: LasI produces 3-oxo-C12-HSL for activation of LasR (Pearson et al., 1994), RhlI produces C4-HSL for the activation of RhlR (Latifi et al., 1995; Pearson et al., 1995), and the biosynthetic enzymes PqsABCDE and PhnAB produce PQS (2-heptyl-3-hydroxy-4-quinolone) for activation of PqsR (Deziel et al., 2004; Gallagher et al., 2002; McGrath et al., 2004). QS systems are also prevalent in many other Gram-negative bacteria, as shown in Table 2 (Dobretsov et al., 2009).

Table 2. QS systems in Gram-negative bacteria

Organism	Autoinducers	Regulated processes, genes and compounds
Aeromonas hydrophila	C4-HSL	Biofilms (Swift et al., 1999)
Aeromonas salmonicida	C4-HSL	Exoproteases (Swift et al., 1997)
Agrobacterium tumefaciens	3-oxo-C8-HSL	Plasmid conjugation (Oger et al., 1998)
Agrobacterium viciae	3-oxo-C16	Virulence (Zhu et al., 1997)
Burkholderia cenocepacia	C6-HSL	Biofilms, swarming (Huber et al., 2001; Tomlin et al., 2005), protease (Lewenza et al., 1999)
Chromobacterium violaceum	C6-HSL	Violacein (McClean et al., 1997), cyanide, exoenzyme (Chernin et al., 1998)
Erwinia chrysanthemi	3-oxo-C6-HSL	Pectinases (Nasser et al., 1998)
Erwinia stewartii	3-oxo-C6-HSL	Exopolysaccharides (Beck von Bodman and Farrand, 1995)
Pseudomonas aeruginosa	3-oxo-C12-HSL	Biofilms (Parsek and Greenberg, 1999), exoenzymes, exotoxins, virulence, pyocyanin(Latifi et al., 1995)
Pseudomonas aureofaciens	C6-HSL	Phenazines (Pierson et al., 1994), proteases (Zhang and Pierson, 2001)
Rhizobium leguminosarum	3-hydro-C8-HSL	Stationary phase adaption (Thorne and Williams, 1999), Root nodulation (Rodelas et al., 1999)
Serratia marcescens	C6-HSL	Prodigiosin (Thomson et al., 2000)
Serratia liquefaciens	C4-HSL	Swarming, exozymes (Eberl et al., 1999; Givskov et al., 1998)
Xenorhabdus nematophilus	3-hydroxy-4-HSL	Virulence (Dunphy et al., 1997)

3. QS systems of Gram-positive bacteria

In contrast to Gram-negative bacteria, many Gram-positive bacteria communicate using modified oligopeptides as signals, and “two-component”-type membrane-bound sensor histidine kinases as receptors. In staphylococci there is only one known system that involves a QS signal, the *agr* QS system, which is composed of the *agrABCD* genes (Novick et al., 1995). The excreted signal is a thiolactone- or lactone-based peptide (AIP, autoinducer peptide) that mediates communication with other staphylococci in a cell density-dependent manner (Ji et al., 1997; Ji et al., 2005; Lyon and Novick, 2004). Excreted AIP reverse-signals via a classical two-component signaling module (AgrC, transmembrane receptor histidine kinase; AgrA, DNA binding response regulator), thereby autoinducing the expression of many extracellular virulence factors in a quorum sensing-dependent manner. At low cell density, the bacteria express protein factors that promote attachment and colonization, whereas at high cell density, the bacteria repress these traits and initiate secretion of toxins and proteases that are presumably required for dissemination. Agr is conserved throughout the staphylococci but has diverged within the genus. The *S. epidermidis* AIP is an octapeptide containing a thiolester linkage between the central cysteine and the C-terminal carboxyl group (Otto et al., 1998), and is active at nanomolar concentrations. Cross-inhibition studies of AIPs between *S. aureus* and *S. epidermidis* revealed that most *S. aureus* subgroups are sensitive towards the *S. epidermidis* AIP. This indicates that quorum-sensing cross talk seems to be generally in favor of *S. epidermidis*, which might explain the predominance of *S. epidermidis* on the skin and in implant-associated infections (Otto et al., 1998). However, AIPs of the *S. intermedius* group species are lactone peptides containing a conserved hydroxyl group on a serine residue. Lactone-peptides are not only used by Staphylococci, but also utilized by *Enterococcus faecalis* to activate gelatinase production, which is a QS-controlled virulence factor (Nakayama et al., 2001).

4. Interphylum interference

Intra-species communication in Gram-positive or Gram-negative bacteria has been well studied. However, most microorganisms constantly have to compete for resources in nature, to prevail against competitors from different species or phylums,

and to survive in hostile environments. Do Gram-positive bacteria or Gram-negative bacteria dominate in specific environments? What happens if staphylococci encounter other bacteria in the host or other environments? One of the most well-known examples is when *S. aureus* encounters *Pseudomonas* in the lungs of cystic fibrosis (CF) patients. According to the Annual Cystic Fibrosis Patient Registry Report, *S. aureus* is the predominant respiratory bacterium in the first ten years of CF patients, after which *P. aeruginosa* gradually catches up as *S. aureus* is simultaneously suppressed. The mechanisms underlying the suppression of *S. aureus* in this co-infection most likely rely on an arsenal of small respiratory inhibitors, such as the phenazine, pyocyanin, hydrogen cyanide or quinoline *N*-oxides excreted by *P. aeruginosa.* These respiratory toxins act both against the commensal flora as well as host cells (Lau et al., 2004). *P. aeruginosa* itself is protected as it possesses a cyanide-insensitive terminal oxidase related to the cytochrome *bd* quinol oxidases (CioAB) (Cunningham et al., 1997). In *S. aureus* and other pathogenic species, the cytochrome *bd* quinol oxidase (CydAB) is inhibited by pyocyanin hydrogen cyanide (Voggu et al., 2006), but it is not completely suppressed, as a sub-population readily adapts to become small colony variants (SCV) that can withstand pyocyanin and hydrogen cyanide exposure (Biswas et al., 2009; Hoffman et al., 2006). These *in vitro* findings correlate well with the clinical outcome, showing that *S. aureus* SCVs that demonstrated hemin, thymidine, and/or menadione dependencies are highly prevalent in the respiratory secretions of CF patients, persist over extended periods, and contribute to *S. aureus* persistence in CF patients (Kahl et al., 1998). The aforementioned examples illustrate self-protection mechanisms that allow some bacterial species not only to prevail but also to persist in unfavorable habitats.

Another method to ensure persistence in natural environments and to limit competition is by excreting antibiotics. This is utilized by some skin staphylococci, which produce so-called lantibiotics such as epidermin or gallidermin (Kellner et al., 1988; Schnell et al., 1988). These lantibiotics inhibit a wide spectrum of Gram-positive bacteria by interacting with lipid I, II, III and IV, thereby inhibiting cell wall and wall teichoic acid (WTA) biosynthesis (Bonelli et al., 2006; Brötz et al., 1998; Müller et al., 2012).

5. Aim of the study

While studying the potential interactions of *Staphylococcus aureus* and *Staphylococcus carnosus* with Gram-negative bacteria (Biswas et al., 2009; Voggu et al., 2006), we came across a communication system in another *Staphylococcus* species group, named 'intermedius group'. This group consists of closely related, mainly coagulase-positive bacterial species, including *S. delphini*, *S. intermedius*, *S. lutrae*, *S. pseudintermedius*, and *S. schleiferi*. They are all zoonotic pathogens, and only rarely infect humans.

In the first part of the study, we found that these species excrete two small compounds that inhibit the expression of QS-controlled toxins and other QS-regulated factors in Gram-negative bacteria.

Our aim was to gain insight into the structure and function of these two compounds.

One of the first milestones was the purification and subsequent identification of the structure of these two excreted compounds in cooperation with Prof. Dr. Stephanie Grond from the organic chemistry department in Tübingen. Once this was achieved, we then sought to chemically synthesize these compounds on a large scale, in order to study their function against Gram-negative bacteria.

This study revealed two excreted compounds (yayurea A and yayurea B) that represent new bacterial products capable of quenching QS regulation in a wide spectrum of Gram-negative bacteria. Furthermore, growth of yayurea A- and B-producing strains is not suppressed by respiratory toxins when co-cultured with *P. aeruginosa.* This suggests that the quorum quenchers have a function in self-protection and competitiveness in natural environments shared with Gram-negatives. We show here an example of inter-phylum interference between firmicutes (Gram-positive bacteria) and the beta- and gamma-proteobacteria (Gram-negative bacteria)

Results

1. Structural elucidation of QS-inhibiting compounds from *S. delphini*

First, we tested the ability of several staphylococcal species onto inhibit pyocyanin production of *P. aeruginosa* pyocyanin production in a co-cultivation assay, as pyocyanin production is QS controlled. We found that *S. delphini* DSMZ 20771 completely inhibited pyocyanin production over 24 h in co-cultivation with *P. aeruginosa* PAO1, while *Staphylococcus aureus* showed no such activity **(Fig. 1A)**. This led us to assess if *S. delphini* could also suppress QS-controlled phenotypes in other Gram-negative bacteria, such as QS-regulated prodigiosin production in *Serratia marcescens* (Thomson et al., 2000); bioluminescence in *Vibrio harveyi* (Anetzberger et al., 2009; Freeman and Bassler, 1999a); or violacein production in *Chromobacterium subtsugae*, a pathogen of potato beetles (Martin et al., 2007). Indeed, in co-cultivation studies with these Gram-negative bacteria, *S. delphini* did also suppress prodigiosin and violacein production, as well as bioluminescence, while *S. aureus* did not **(Fig. 1BCD)**. Sterile filtered culture supernatant of a 24 h *S. delphini* culture had the same QS-inhibiting effect as the co-culture, indicating that the QS-inhibiting compound(s) were excreted. The supernatants of *S. aureus* and *S. epidermidis* caused no effect.

To determine the approximate molecular mass of excreted compound(s), filtered supernatants of *S. aureus* and *S. delphini* were passed through molecular mass cut-off filters and analyzed for pigment suppression activities in *S. marcescens*. The supernatant from *S. delphini* maintained activity after filtration through a 3-kDa molecular weight cut-off membrane **(Fig. 2)**. To derive more chemical properties, the supernatant was further treated with heat, proteinase K, acid, or alkali for 1 h. However, the activity was not affected by any treatment **(Fig. 2)**, demonstrating that the QS inhibitor(s) in question are small (below 3 kDa), pH- and heat-stable compound(s) that may not be protein-based.

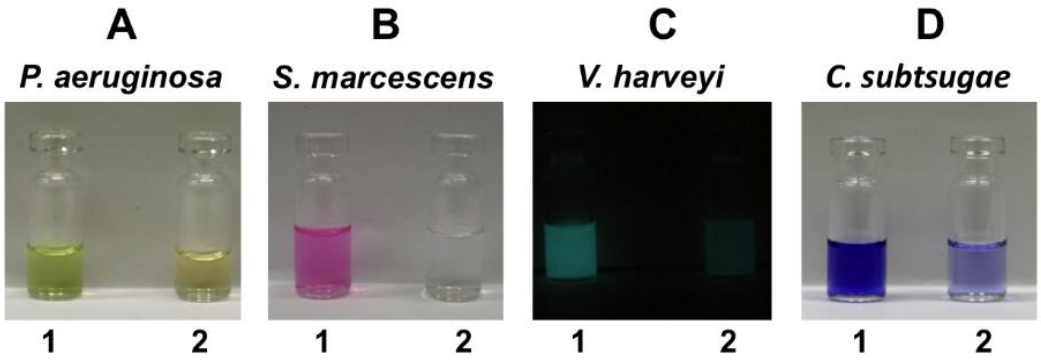


Figure 1. Quenching of QS-regulated pigments and bioluminescence by *S. delphini*

P. aeruginosa (**A**), *S. marcescens* (**B**), *V. harveyi* (**C**) and *C. subtsugae* (**D**) were each co-cultivated with *S. aureus* (1) or *S. delphini* (2) for 24 h. Pyocyanin, which is excreted by *P. aeruginosa*, was assessed in the supernatant at its absorption maximum A_{520nm}. Prodigiosin, which is cell wall-bound in *S. marcescens*, was ethanol-extracted from the cell pellet and determined at its absorption maximum A_{534nm}. Bioluminescence of *V. harveyi* was intensified by aeration before measurement in a bioluminescence reader. Violacein from *C. subtsugae* was quantitatively extracted with butanol and assessedd at its absorption maximum A_{585nm}.

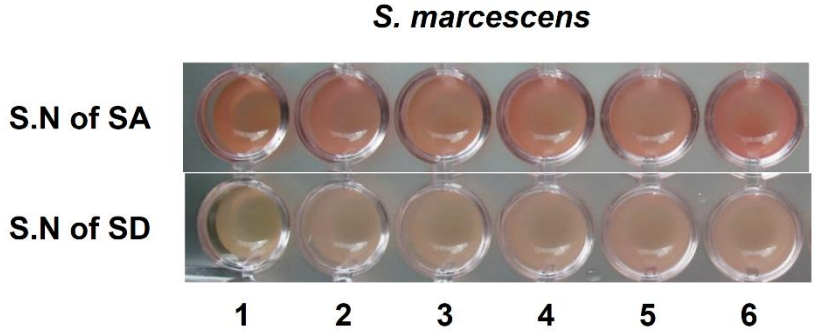


Figure 2. Characterization of the active compounds of *S. delphini* supernatant

S. aureus (SA) and *S. delphini* (SD) were cultured in TSB for 24 h. The supernatants were collected (**1**) and dialyzed with 3-kDa cutoff membrane (**2-6**). The supernatants were further treated with heat (95 ^{o}C) (**3**), 4 µg/µl Proteinase K (**4**), 10N HCl (**5**) or 10N NaOH (**6**) for 1 h. All supernatants had pH values adjusted to 7, and were incubated with *S. marcescens* for 24 h.

Reversed phase preparative (RP-HPLC) analysis of *S. delphini* supernatant showed the active compound(s) had a retention time (Rt) of 4 to 5 minutes which contained

two absorption signals at 210 nm **(Fig. 3)**. At the same time, comparison of the RP-HPLC profiles of *S. aureus* with *S. delphini* showed that these two absorption peaks (peak 2 and 4) were only seen in the supernatant of *S. delphini* **(Fig. 4).** The data implies that these two absorption peaks might be the QS inhibitors in question. To purify the active compounds, several purification steps were performed **(Fig. 5 and 6)**. The purification process revealed that the supernatant contained two compounds with different retention times (R_t) in HPLC and distinct UV spectra. We subsequently named the two compounds yayurea A and B.

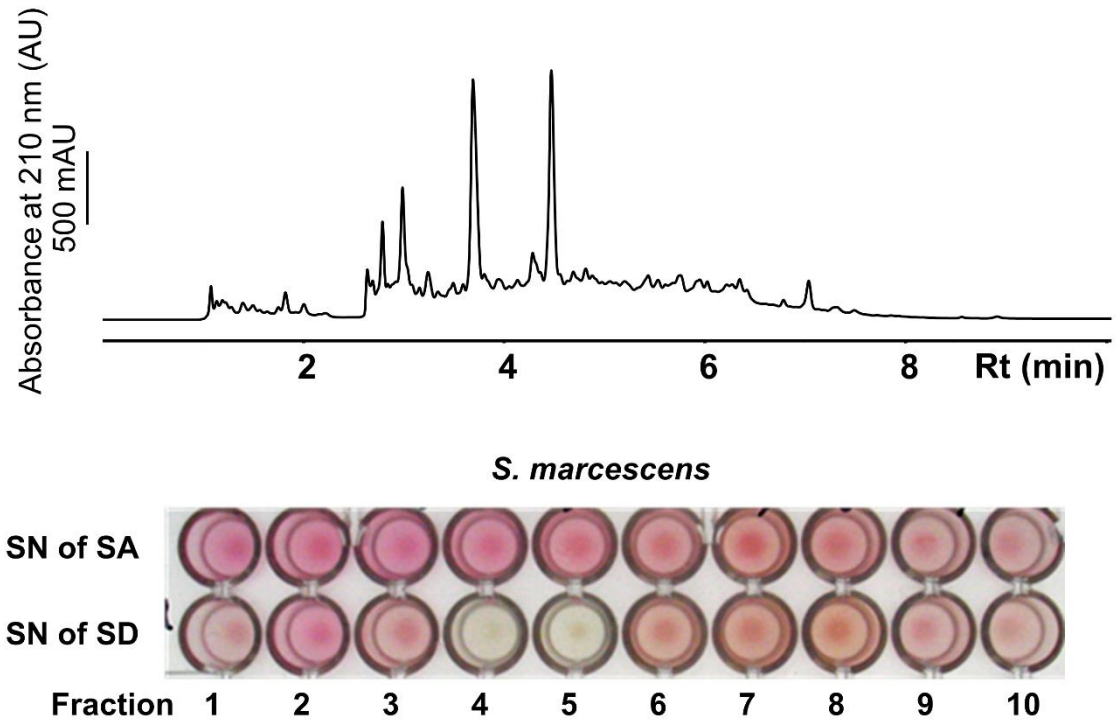


Figure 3. Location of the active compound within the HPLC profile of *S. delphini* supernatant
The supernatant of *S. delphini* was analyzed and separated into 10 fractions by RP-HPLC. The collected fractions were lyophilized, resuspended by water and inoculated with *S. marcescens* for 24 h.

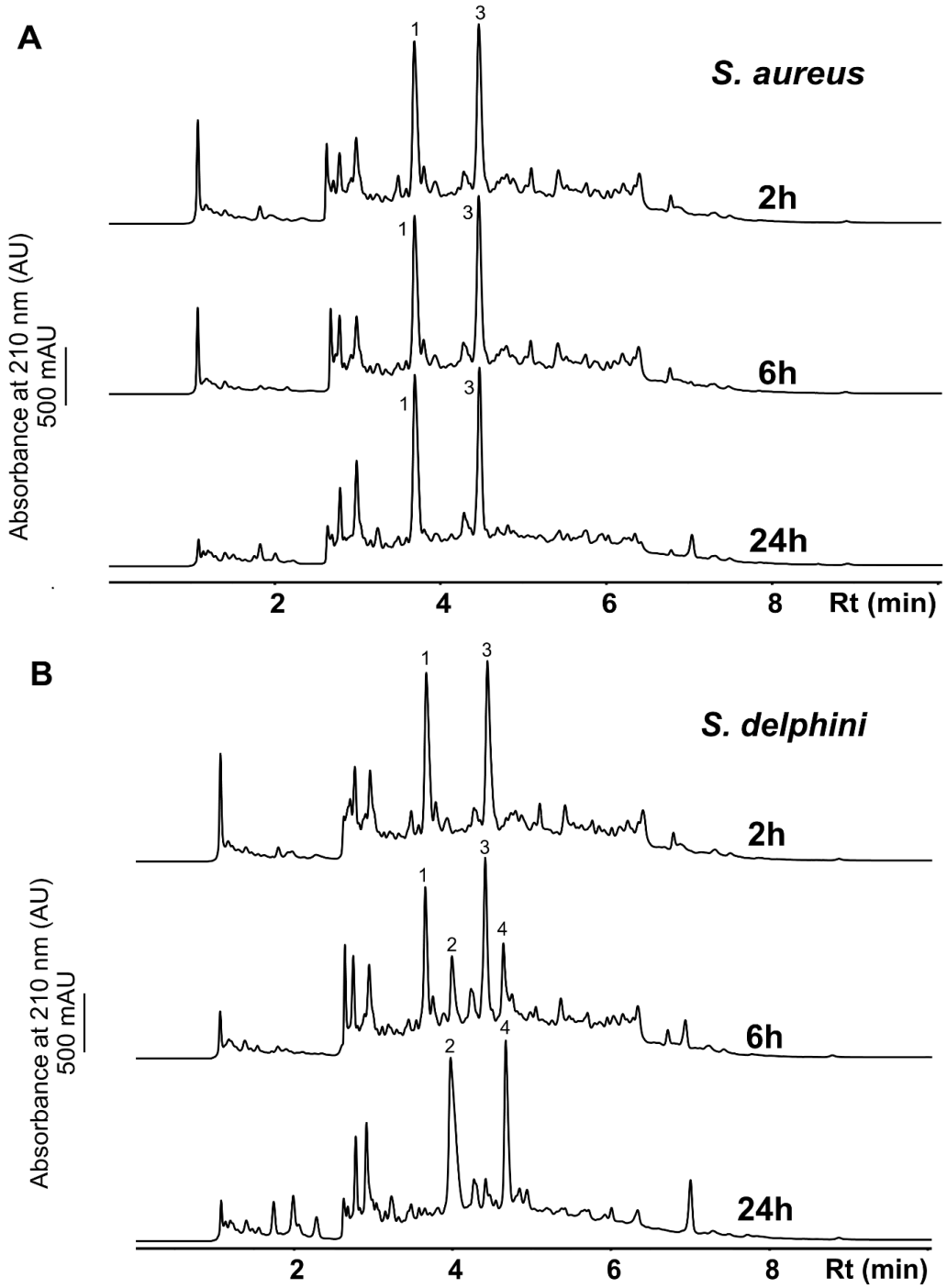


Figure 4. RP-HPLC profile comparison of S. aureus and S. delphini
RP-HPLC profiles of the culture supernatants of *S. delphini* (**B**) showing the production progression of yayurea B and yayurea A (peaks 2 and 4) at 2 h, 6 h and 24 h in comparison to the non-producing *S. aureus* (**A**). HPLC analysis was carried out on an Agilent 1200 and Waters xBridge C18, 5 µm, 4.6x150 mm column. Elution was made with a 15 min linear gradient of 0.1% Phosphoric to acetonitrile at a flow rate of 1.5 ml/min.

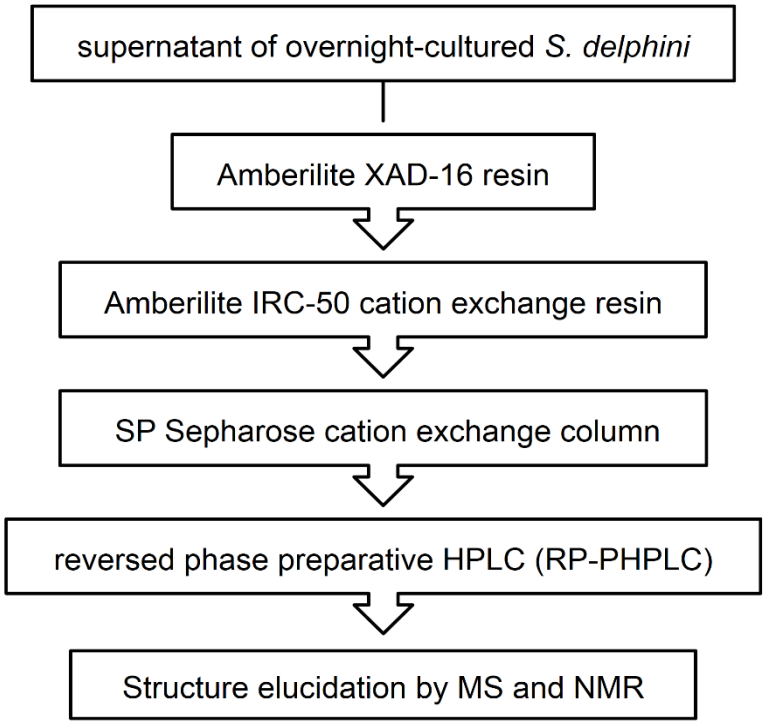


Figure 5. Outline of the compound purification procedures
The supernatant of *S. delphini* was collected and applied to different resins in the order shown above. The elutions of each step were collected and tested for QS inhibition activity with *S. marcescens.* The fractions with no QS inhibition activity were discarded; the fractions with QS inhibition activity were moved on to the following procedure.

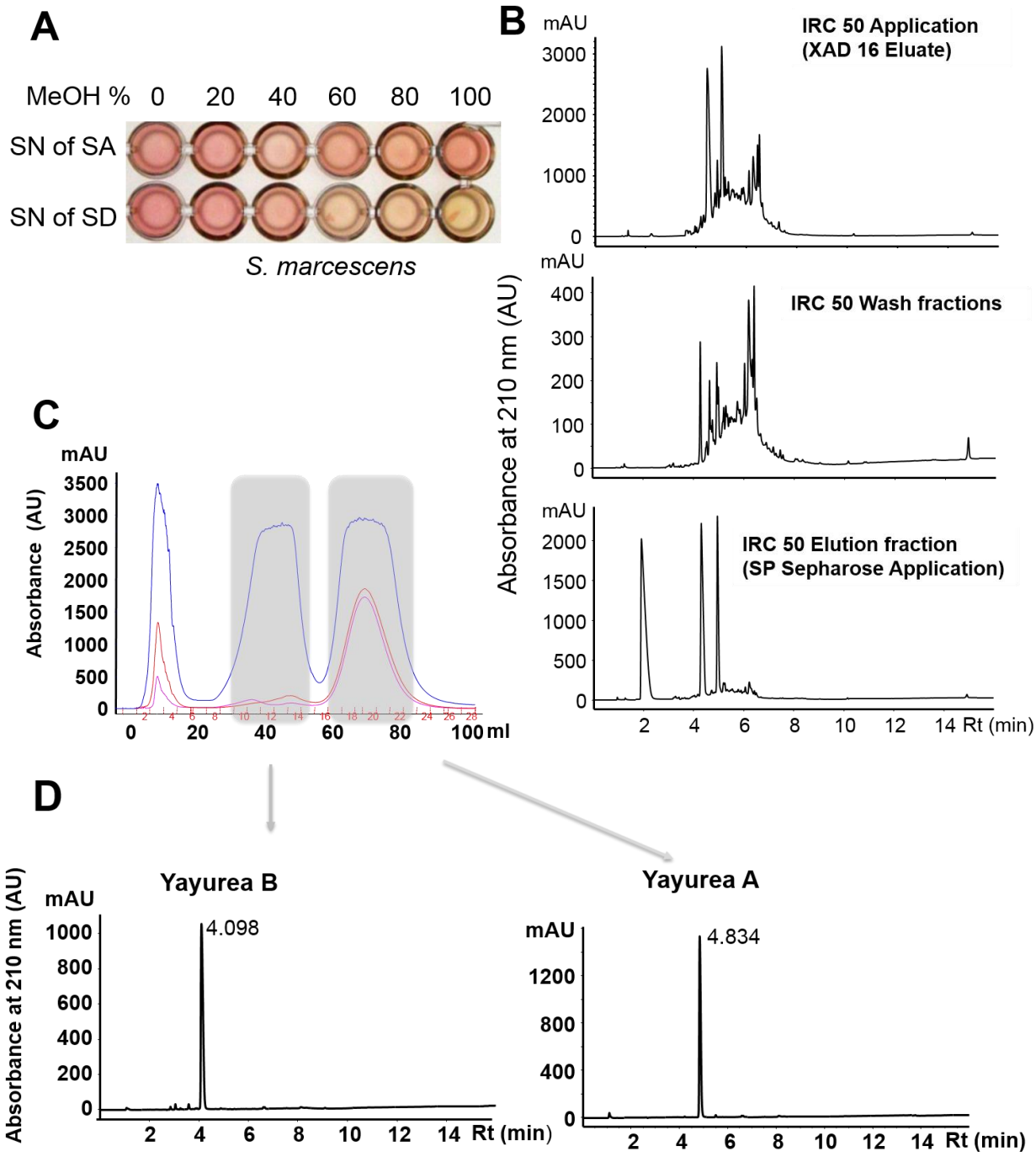


Figure 6. Purification of QS-inhibitors

(**A**) *S. delphini* supernatant was applied to a column filled with an Amberilite XAD-16 resin. The column was washed with water and eluted with 0% to 100% MeOH. The collected fractions were lyophilized, resuspended by water, and inoculated with *S. marcescens* for 24 h. (**B**) The eluates by 80% MeOH and 100% MeOH from XAD-16 resin were applied to a column filled with Amberilite IRC-50 cation exchange resin. The column was washed with 70% EtOH and eluted with 80% EtOH acidified with 5% acetic acid. (**C**) The eluate was further separated on a SP Sepharose cation exchange column with a linear 1 M NaCl gradient in 50 mM sodium phosphate buffer.

The two active compounds were eluted separately. (**D**) The final purification and desalting of each peak was carried out by RP-HPLC on a nucleosil 100 C-18, 8×250 mm column with a linear water acetonitrile (containing 0.1% TFA) gradient of 0% to 60% in 25 min. (M. Nega, MSc thesis)

Yayurea A (indole-ethylurea) was isolated as a brownish solid, and revealed an ion peak at m/z = 161 ($[M+H]^+$) in ESI-MS **(Fig. 7A)**, while the molecular formula, $C_{10}H_{13}N_2$, was revealed in FT-ICR-MS analysis. The 1*H*-indole-3-ethylamine moiety was deduced from GC-EI-MS (R_t = 29.0 min). ^{1}H-NMR spectra showed a characteristic singlet at δ_H = 8.55 ppm **(Fig. 8A)**. The remaining signals were assigned to 2-(3-indoyl) ethylamine. The ^{13}C-NMR spectrum and additional HSQC experiments displayed signals pointing to a carbonyl group at δ = 170.5 ppm **(Fig. 8B)**, five methin groups, and two methylene groups to reveal a urea moiety. Additionally, 2D NMR experiments supported the structure of yayurea A as *N*-[2-(1H-indole-3-yl) ethyl]-urea **(Fig. 9A)**. Yayurea B (phenethylurea) was obtained as a colorless solid. A preliminary molecular formula, $C_8H_{10}N$, was deduced from the FT-ICR-MS spectrum, which showed an ion at m/z = 121 ($[M+H]^+$) **(Fig. 7B)**. GC-EI-MS provided a signal at R_t = 15.0 min pointing to 2-phenethylamine. The ^{1}H-NMR again showed a set of aromatic protons and a singlet at δ_H = 8.54 ppm **(Fig. 8C)**, while the ^{13}C-NMR displayed a signal at δ_H = 170.4 ppm (C=O) and five aromatic and 4 aliphatic protons **(Fig. 8D)**. In summary, the structure of *N*-(2-phenethyl)-urea was assigned for yayurea B **(Fig. 9B)**. UV-absorption maxima emphasized the phenyl (λ = 260 nm) and indole chromophores (λ = 225 nm and λ = 280 nm respectively) of the yayureas. Comprehensive physicochemical characteristics of yayurea A and B are shown as follows **(Table 3)**.

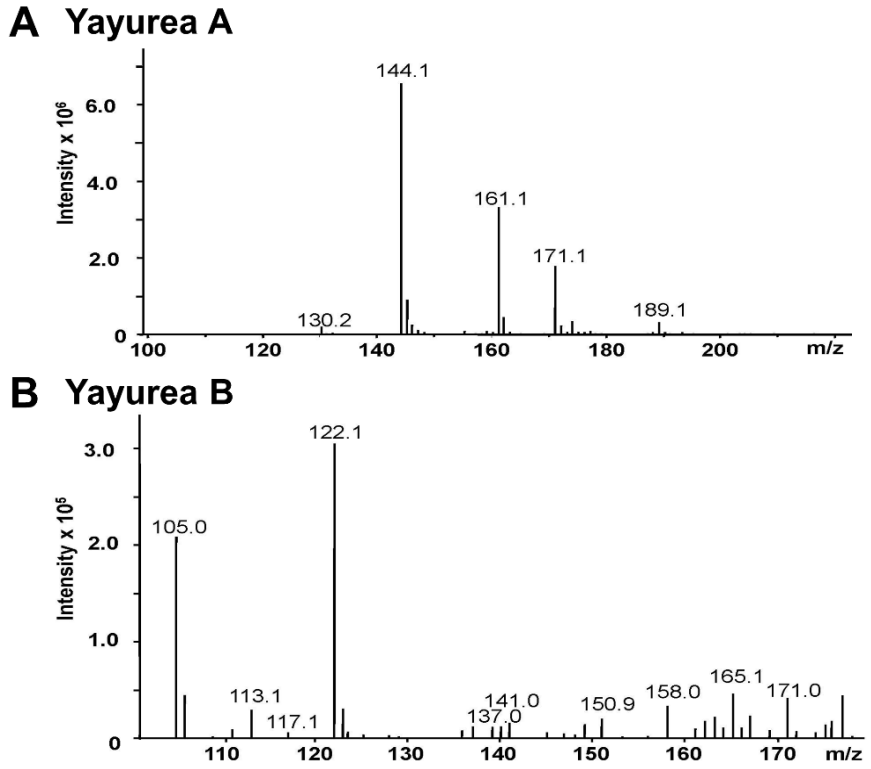


Figure 7. Mass spectra of the two QS-inhibitors purified from *S. delphini*
Mass spectrometry was carried out on GC-MS and FT-ICR MS (Bruker, ApexII).

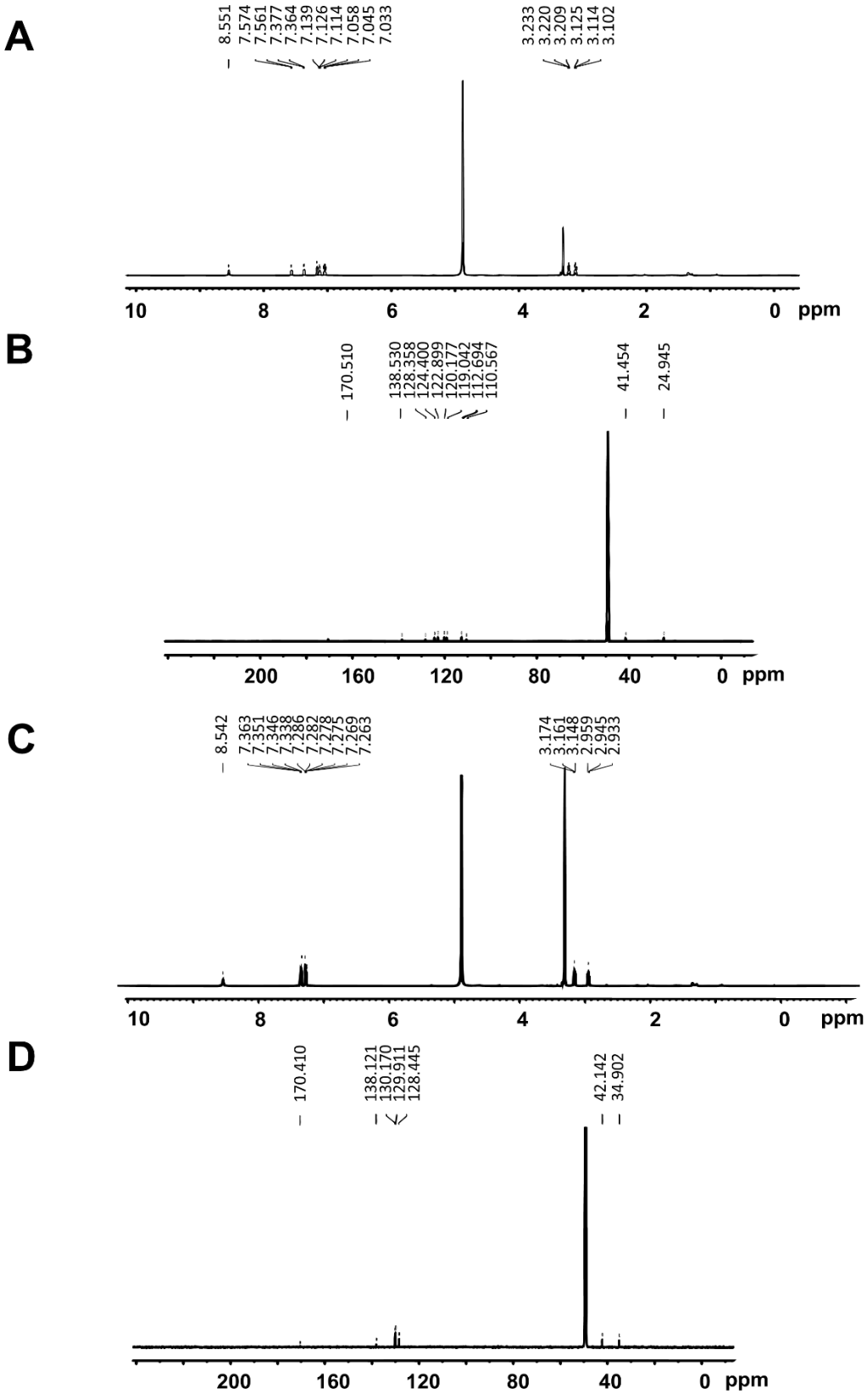


Figure 8. NMR spectra of the QS inhibitors purified from *S. delphini*

^{1}H-NMR-spectrum (600 MHz, MeOD) of yayurea A (**A**) and yayurea B (**C**). ^{13}C-NMR-spectrum (150 MHz, MeOD) of yayurea A (**B**) and yayurea B (**D**). (Wöfle *et al.* unpublished).

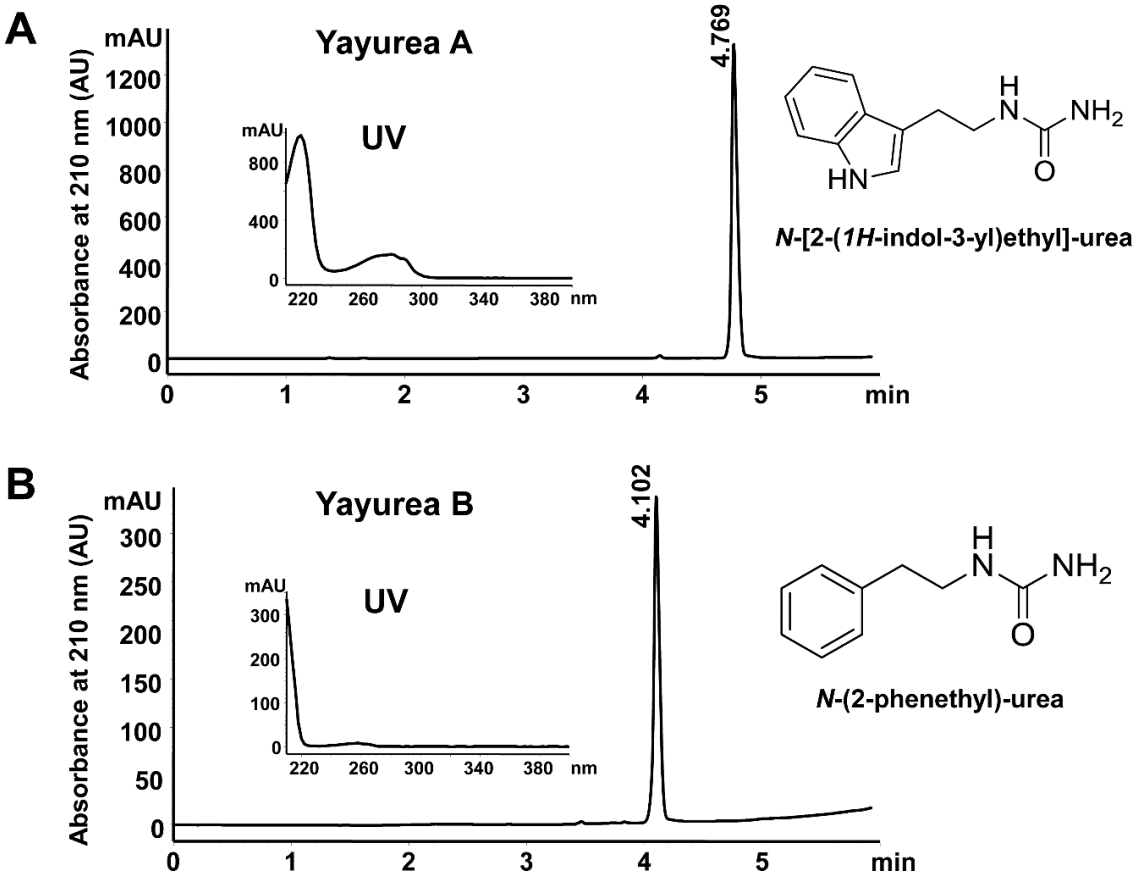


Figure 9. RP-HPLC profile, UV spectrum, and structures of the two QS-inhibitors purified from *S. delphini*

(**A**) QS-inhibitor, *N*-[2-(1H-indol-3-yl)ethyl]-urea (yayurea A). (**B**) QS-inhibitor, *N*-(2-phenethyl)-urea (yayurea B). RP-HPLC was carried out on an Agilent 1200 and Waters xBridge C18, 5 mm, 4.6x150 mm column; compounds were eluted with a 15 min linear gradient of 0.1% phosphoric acid to acetonitrile at a flow rate of 1.5 ml/min.

Table 3. Physicochemical characteristics of yayurea A and B

	***N*-[2-(*1H*-indol-3-yl)ethyl]-urea (yayurea A)**	***N*-(2-phenethyl)-urea (yayurea B)**
Formula	**$C_{11}H_{13}O_1N_3$** (203.24 g/mol)	**$C_9H_{11}O_1N_2$** (163.20 g/mol)
Melting point	240.8 °C	214.9 °C
R_f-values	0.10 ($CHCl_3$/MeOH 9:1) 0.63 (MeOH/H_2O 7:3)	0.28 ($CHCl_3$/MeOH 9:1) 0.94 (MeOH/H_2O 7:3)
^{1}H NMR	(600 MHz, MeOH-d_4) d 3.11 (t, J = 7.0, 7.4 Hz, 2H), 3.22 (t, J = 7.1, 7.3 Hz, 2H), 7.04 (dd, J = 7.4, 7.5 Hz, 1H), 7.13 (dd, J = 7.7, 7.9 Hz, 1H), 7.17 (s, 1H), 7.37 (d, J = 8.2 Hz, 1H), 7.57 (d, J = 7.9 Hz, 1H), 8.55 (s, 1H).	(600 MHz, MeOH-d_4) d 2.95 (t, J = 7.4, 8.1 Hz, 2H), 3.16 (t, J= 7.4, 8.1 Hz, 2H), 7.28 (m, 3H), 7.35 (m, 2H), 8.54 (s, 1H).
^{13}C NMR	(150 MHz, MeOH-d_4) d 24.9, 41.5, 110.6, 112.7, 119.0, 120.2, 122.9, 124.4, 128.4, 138.5, 170.5.	(150 MHz, MeOH-d_4) d 34.9, 42.1, 128.4, 129.9, 130.2, 138.1, 170.2.
MS (ESI)	(Positive ions) *m/z* (%) [M+2H-$CONH_2$]$^+$ 161.11.	(Positive ions) *m/z* (%) [M+2H-$CONH_2$]$^+$ 122.10.

Yayurea A and B were also chemically synthesized and their identities confirmed by mass spectrometry and NMR analyses (Wölfle *et al*. manuscript in preparation). Both the synthesized and the natural compounds revealed the same chemical properties, such as retention time and UV absorption **(Fig. 10 and 11)**, and had the same QS-quenching activity in Gram-negative bacteria (data not shown).

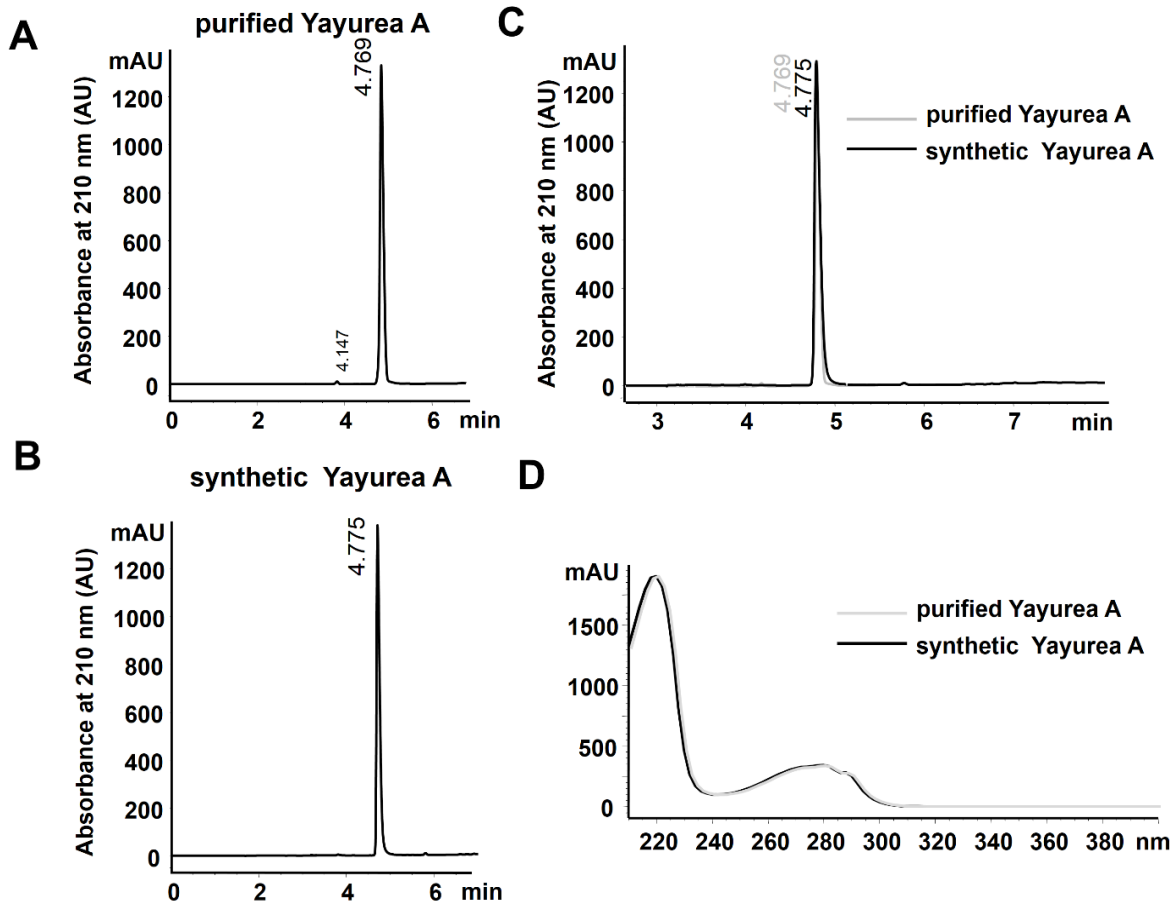


Figure 10. Comparison of purified and synthetic yayurea A

The RP-HPLC profiles of purified (**A**) and synthetic (**B**) yayurea A. The overlapped RP-HPLC (**C**) and UV-Vis spectrum (**D**).

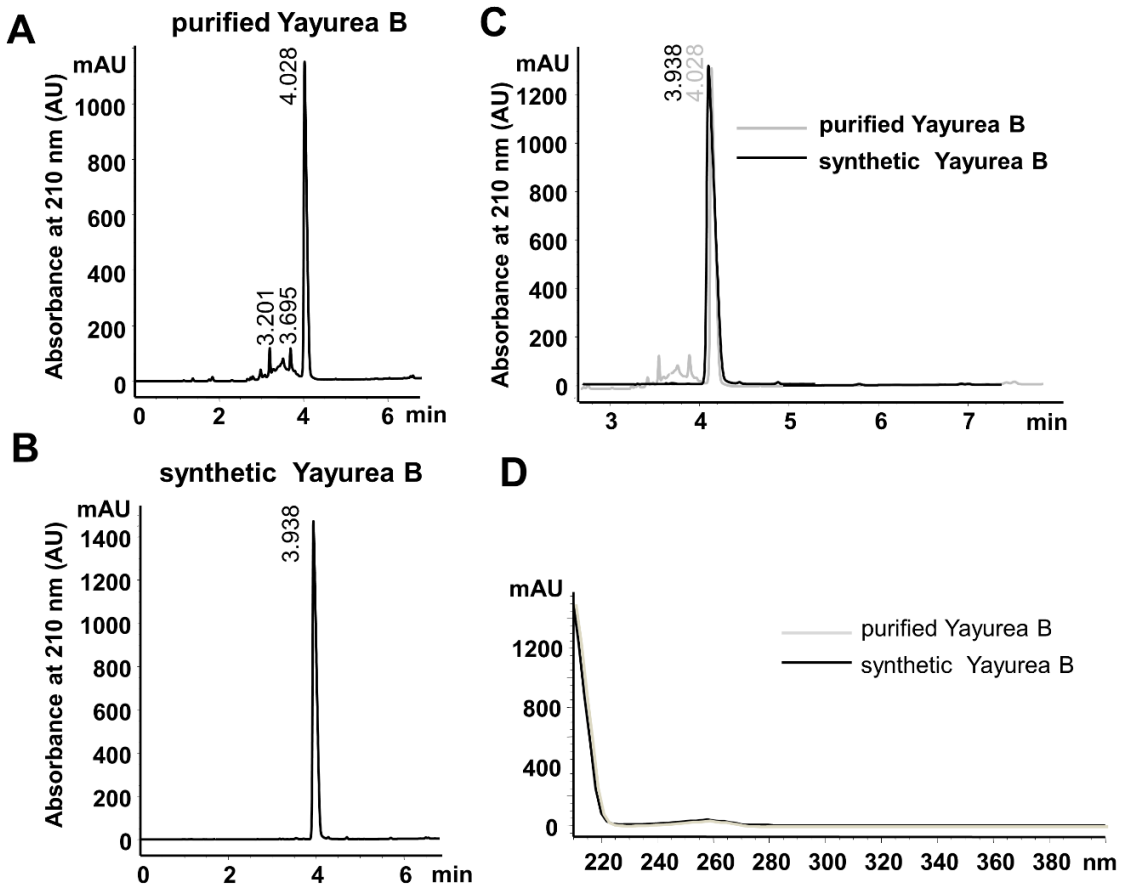


Figure 11. Comparison of purified and synthetic yayurea B
The RP-HPLC profiles of purified (**A**) and synthetic (**B**) yayurea B. The overlapped RP-HPLC (**C**) and UV-Vis spectrum (**D**).

In **Fig. 4**, both *S. aureus* and *S. delphini* showed absorption peak 1 and 3 at the beginning of the culture. The amounts of peak 1 and peak 3 were not changed in *S. aureus*, but were reduced in *S. delphini* over time. Interestingly, as peak 1 and peak 3 decreased, yayurea A and B (peak 4 and 2 respectively) increased **(Fig. 4B)**. We speculated if the peak 1 and 3 were precursors for the yayureas? After separation, these two peaks were analyzed by GC-MS and FT-ICR MS. The results of MS showed that peak 1 is phenylalanine and peak 3 is tryptophan, which were already present in the culture medium (data not shown). To verify the above hypothesis, *S. delphini* was fed with phenylalanine, tryptophan, or tyrosine after 6h, and the production of yayureas were quantified until 72 h. Yayurea A increased up to 300% and 400% with 0.1 mg/ml and 0.3 mg/ml tryptophan, but did not change with phenylalanine or tyrosine administration **(Fig.12A)**. In contrast to yayurea A, adding

0.1 mg/ml and 0.3 mg/ml phenylalanine enhanced yayurea B to 180% and 240%, respectively **(Fig.12B)**.

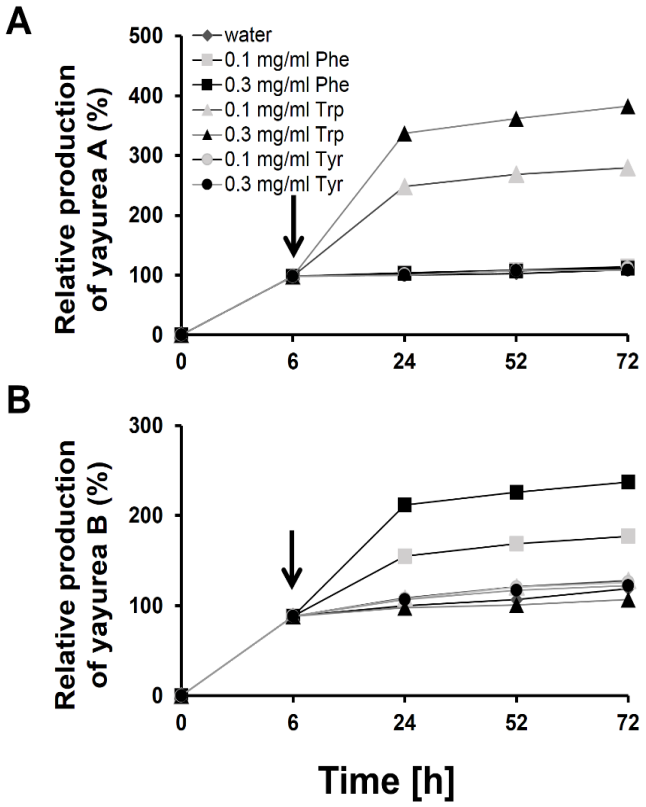


Figure 12. Precursor analysis of yayureas
S. delphini was grown in TSB at 37°C. Cultures were fed with phenylalanine (Phe), tryptophan (Trp), or tyrosine (Tyr) in different concentrations after 6 h. Arrows indicate time point of amino acid feeding. Water was used as a control and the yayurea amount at 24 h was set as 100%. Production of yayurea A (**A**) and B (**B**) were measured by HPLC.

2. Biological properties of yayureas

a) Suppression of respiratory toxins protects from killing by *P. aeruginosa*

P. aeruginosa produces various QS-controlled respiratory toxins, such as pyocyanin and hydrogen cyanide (Castric, 1975; Hassan and Fridovich, 1980), which kill *S. aureus* (Biswas et al., 2009). If yayurea A and B repress the production of QS-regulated pyocyanin or hydrogen cyanide, one would expect that *S. delphini* survives better in a co-culture with *P. aeruginosa* than, for example, *S. aureus*. Indeed, co-cultivation studies with *S. aureus* (non-producer) or *S. delphini* with *P. aeruginosa*

SH1 revealed that the viability (CFU) of *S. aureus* significantly decreased in the stationary growth phase, most likely due to the respiratory toxins produced by *P. aeruginosa* **(Fig. 13A)**, while that of *S. delphini* was uninfluenced **(Fig. 13B)**. The addition of yayurea A and B to the mixed *S. aureus* and *P. aeruginosa* culture protected *S. aureus* from killing in the stationary phase **(Fig. 13A)**. Furthermore, the CFU of *P. aeruginosa* SH1 was unaffected when co-cultured with *S. delphini* or *S. aureus*, indicating that none of the two staphylococcal species was able to kill *P. aeruginosa*. Co-cultivation of *P. aeruginosa* with *S. aureus* in the presence of yayureas (100 µg/ml yayurea A and 900 µg/ml yayurea B) also had no effect on the viability of *P. aeruginosa* **(Fig 13C)**. Other tested *Pseudomonas* strains, PAO1 and DSMZ 50071, showed similar results (data not shown). All in all, these results showed that production of yayurea A and B enables staphylococci to coexist with Gram-negative bacteria in a mixed community.

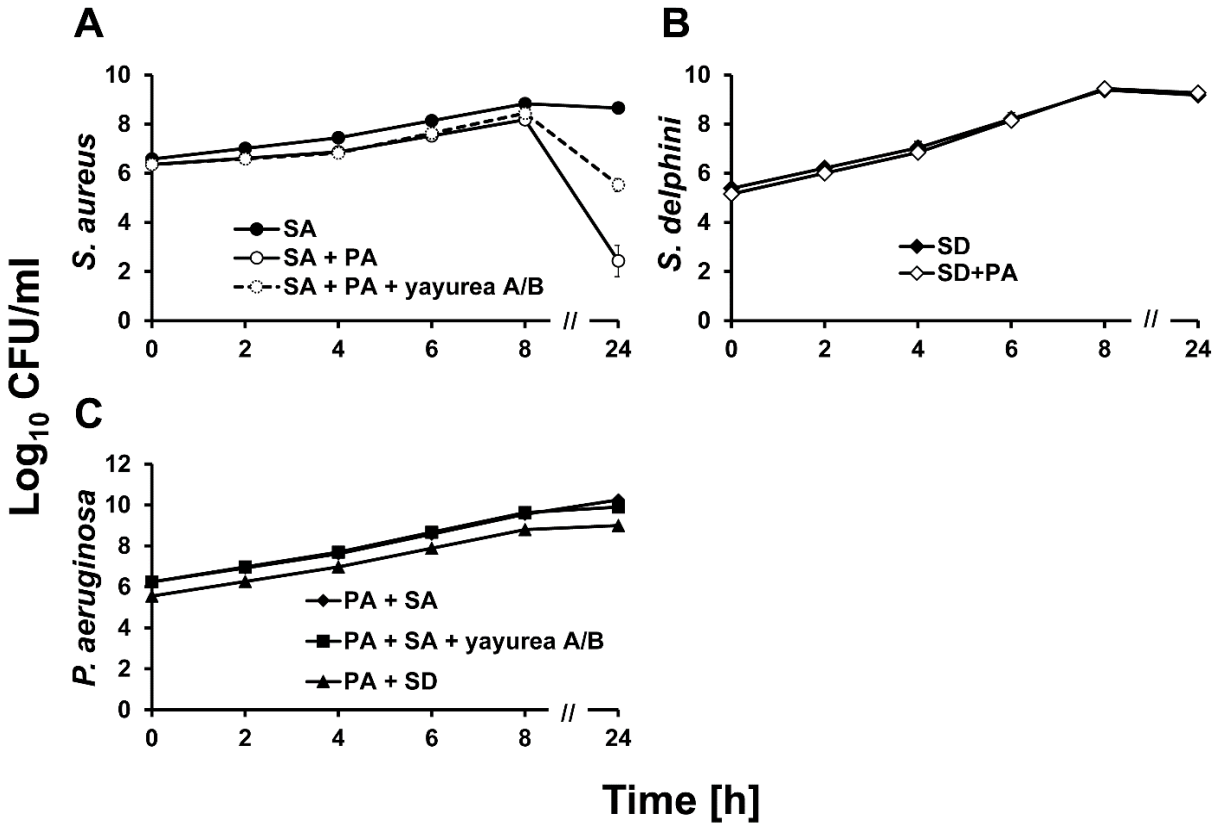


Figure 13. Survival of staphylococcal strains in mixed culture with *P. aeruginosa*

(**A**) CFU of *S. aureus* alone (SA) and in co-culture with *P. aeruginosa* (SA+PA). For the protection test, yayurea A (100 µg/ml) and B (900 µg/ml) were added to the mixture of *S. aureus* and *P. aeruginosa* (SA+PA+yayurea A/B). (**B**) CFU of *S.*

delphini alone (SD) and in co-culture with *P. aeruginosa* (SD+PA). (**C**) CFU of *P. aeruginosa* SH1 co-cultured with *S. aureus* (PA+SA), yayureas (PA+SA+yayurea A/B) or *S. delphini* (PA+SD). Values represent the means of three independent experiments. Bars indicate mean standard deviation, SD.

b) Yayurea A and B are mainly produced in stationary growth phase

We followed the production of yayurea A and B over 24 h in the supernatant of *S. delphini* cultured in TSB. Production levels of yayurea A and B were determined by HPLC-analysis; peak integration was correlated with standard yayurea A and B. The production of both compounds started at the transition of exponential to stationary growth phase (after approximately 4 h) and increased rapidly for the next 5 h; after 24 h little was produced **(Fig. 14)**. The production kinetics are reminiscent of QS-controlled expression. Both compounds were produced in amazingly high concentrations: yayurea A reached concentrations of 120 µg/ml and yayurea B reached levels up to 900 µg/ml. This high concentration is entirely sufficient to suppress QS systems in Gram-negative bacteria, as can be seen below.

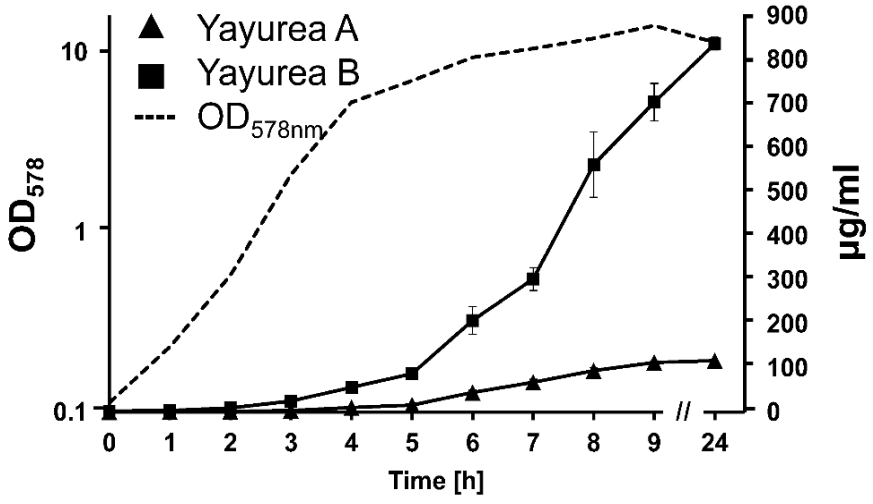


Figure 14. Quantification of yayurea A and B production in relation to growth *S. delphini* was grown in TSB at 37°C. Supernatant was collected and OD_{578} was measured hourly for the first 9 h and after 24 h. Amounts of yayurea A and B in supernatants were quantified by triplicate HPLC measurements. Bars indicate mean standard deviation, SD.

c) Yayurea A is more active in inhibiting various QS-controlled traits in Gram-negative

Purified yayurea A and B inhibited QS-regulated factors in Gram-negative bacteria in a dose-dependent manner. We tested prodigiosin production in *S. marcescens*, bioluminescence in *V. harveyi*, and pyocyanin production in *P. aeruginosa* **(Fig. 15 and 16)**. Yayurea A and B inhibited production of prodigiosin in *S. marcescens* in a dose-dependent manner. Inhibition began at low concentrations (15 μg/ml) and increased with rising concentrations of yayurea A or B. At 250 μg/ml, prodigiosin production was inhibited at 60% (yayurea A) and 40% (yayurea B). At a concentration of 1,000 μg/ml, prodigiosin production was completely inhibited by yayurea A, and approximately 70% by yayurea B **(Fig. 15A)**. At 500 μg/ml, bioluminescence *of V. harveyi* was inhibited by yayurea A and B by 99% and 76%, respectively **(Fig. 15B)**. We also investigated whether yayurea A and B inhibited biofilm formation in *P. aeruginosa* and *S. aureus*. Again, yayurea A quite efficiently inhibited biofilm formation in *P. aeruginosa*, while yayurea B was less effective **(Fig. 15C)**. In contrast to gallidermin, a proven biofilm inhibitor in staphylococci (Saising et al., 2012), both yayureas showed no biofilm-inhibiting effect with *S. aureus* **(Fig. 15D)**.

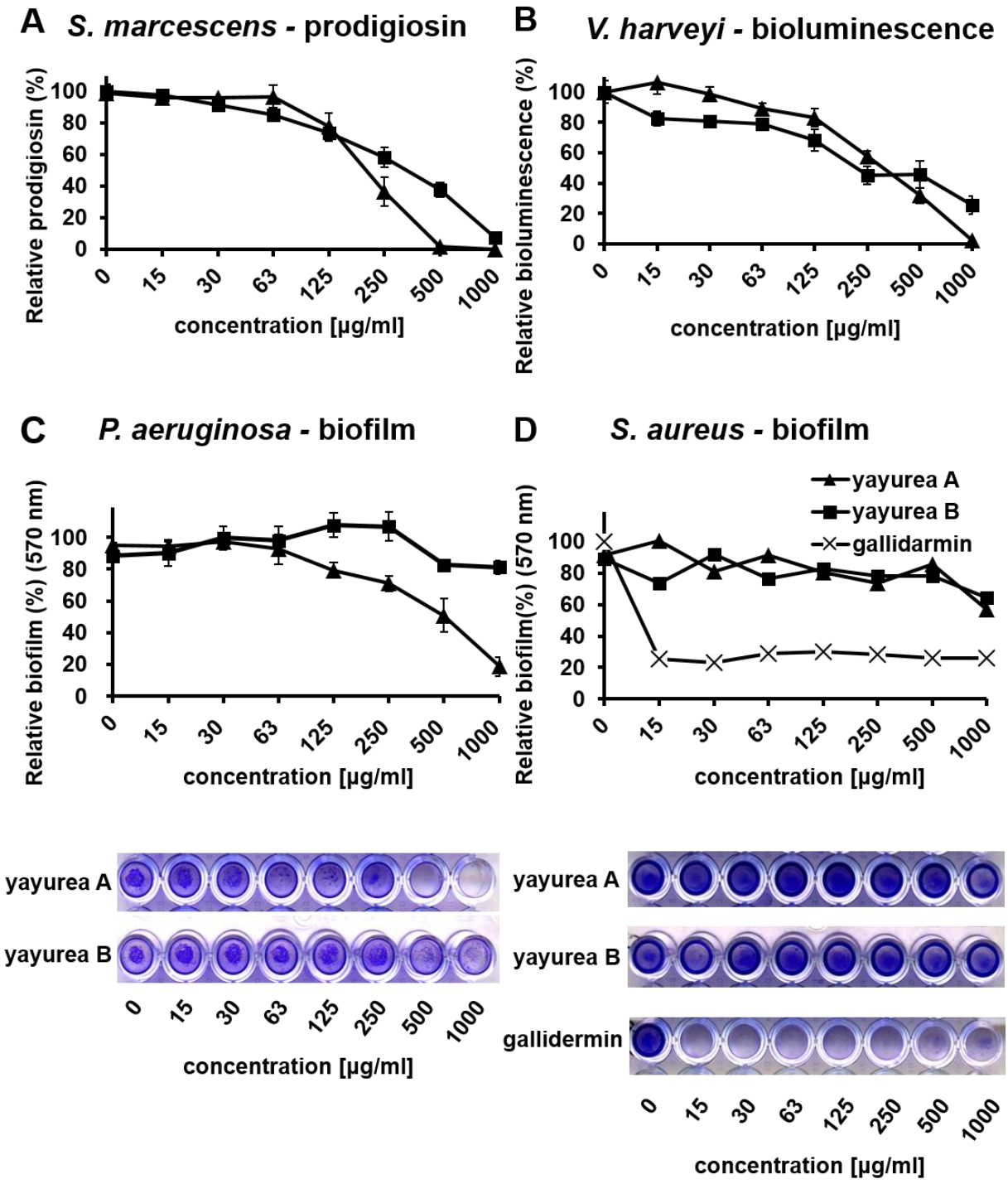


Figure 15. Concentration-dependent inhibition of QS-regulated phenotypes in Gram-negative bacteria

(**A**) Prodigiosin production in *S. marcescens.* Cells were grown in MB medium with serial dilutions of yayurea A or B at 28°C. Relative prodigiosin production was calculated as the ratio between prodigiosin content (absorbance at 534 nm) and cell density (absorbance at 600 nm). (**B**) Bioluminescence in *V. harveyi.* Cells were grown in marine broth with serial dilutions of compounds at 28°C for 24 h. Relative luminescence units were normalized by cell density. (**C**) Biofilm formation of *P. aeruginosa*. Cells were grown in LB with serial dilutions of yayurea A or B at 37°C for

24h. (**D**) Biofilm formation of *S. aureus*. Cells were grown in TSB with serial dilutions of yayurea A, B, or gallidermin (positive control) at 37°C for 24 h. Biofilm cell layers were visualized by crystal violet staining and measured at 590 nm. Microtiter plates presented are representative of at least three independent sets of experiments. Bars indicate standard deviation of the mean, SD.

d) Yayureas have antimicrobial effect

We noticed that high doses of yayurea A and B (especially yayurea A) inhibited the growth of Gram-negative bacteria. We used *P. aeruginosa* as an example to verify the effect of growth and QS inhibition by yayurea A and B, and used the antibiotic tetracycline and the well-known QS-inhibitor furanone (Hentzer et al., 2003) as controls **(Fig 16)**. For QS inhibition, only 50% of the yayurea A and B concentration was necessary, as compared to concentrations needed for growth inhibition **(Fig. 16 A, B)**. Furanone revealed a similar correlation of growth and QS inhibition; concentrations (31 μg/ml) that inhibited 40% of growth inhibited 80% of QS **(Fig. 16C)**. In contrast to yayurea and furanone, tetracycline inhibited growth and QS in a near-linear fashion **(Fig. 16D)**. Besides *P. aeruginosa,* yayureas also affected growth in *S. marcescens, V. harveyi,* and *V. cholerae* **(Fig. 17)**; however, the growth of *E. coli* was not affected. All staphylococcal species tested, such as *S. aureus*, *S. carnosus*, *S. delphini* or *S. schleiferi*, were resistant to yayurea A and B, which was independent of their respective ability to produce these compounds.

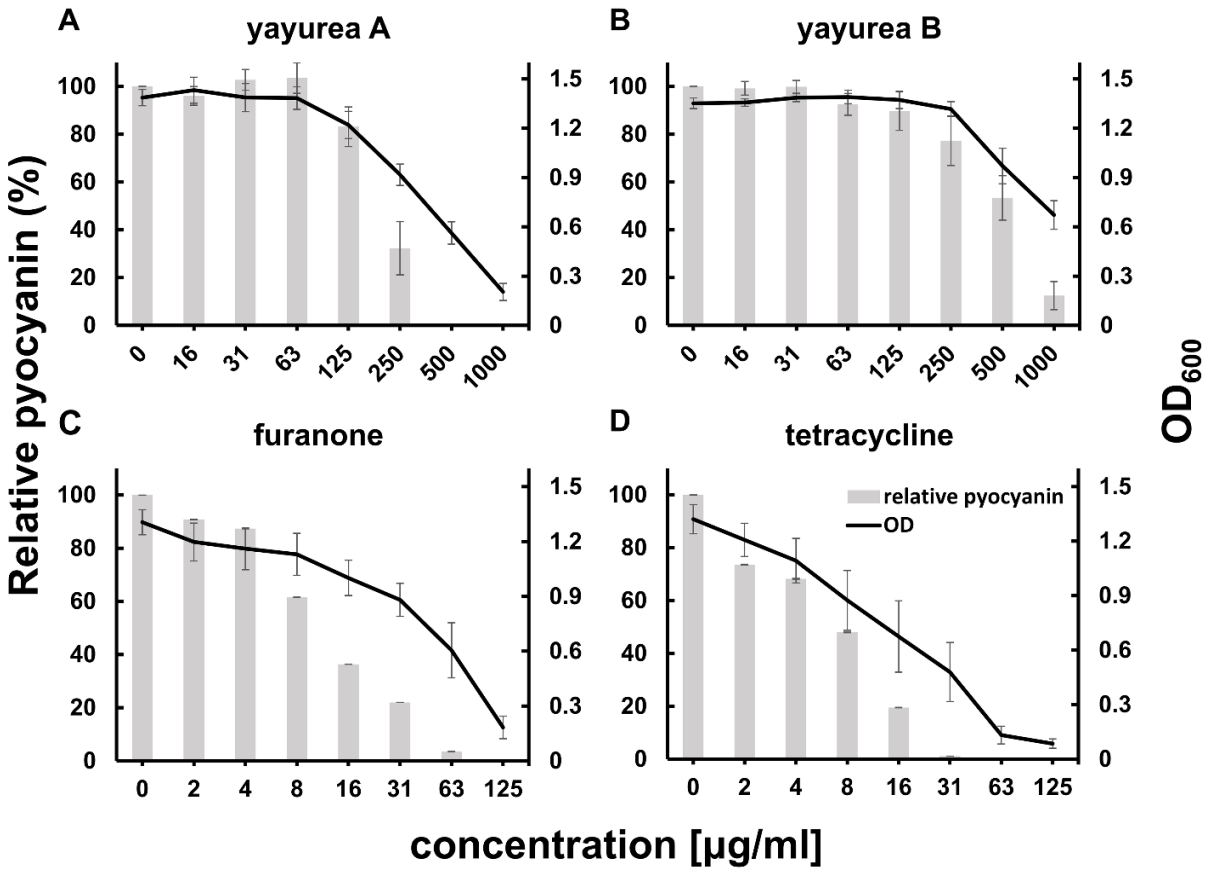


Figure 16. Concentration-dependent inhibition of pyocyanin production and growth of *P. aeruginosa*

P. aeruginosa PAO1 was grown in LB at 30°C with serial dilutions of yayurea A (**A**), yayurea B (**B**), furanone (**C**) and tetracycline (**D**). Relative pyocyanin production was calculated as the ratio between pyocyanin content and cell density (absorbance at 600 nm). Values represent the means of three independent experiments. Bars indicate standard deviation of the mean, SD.

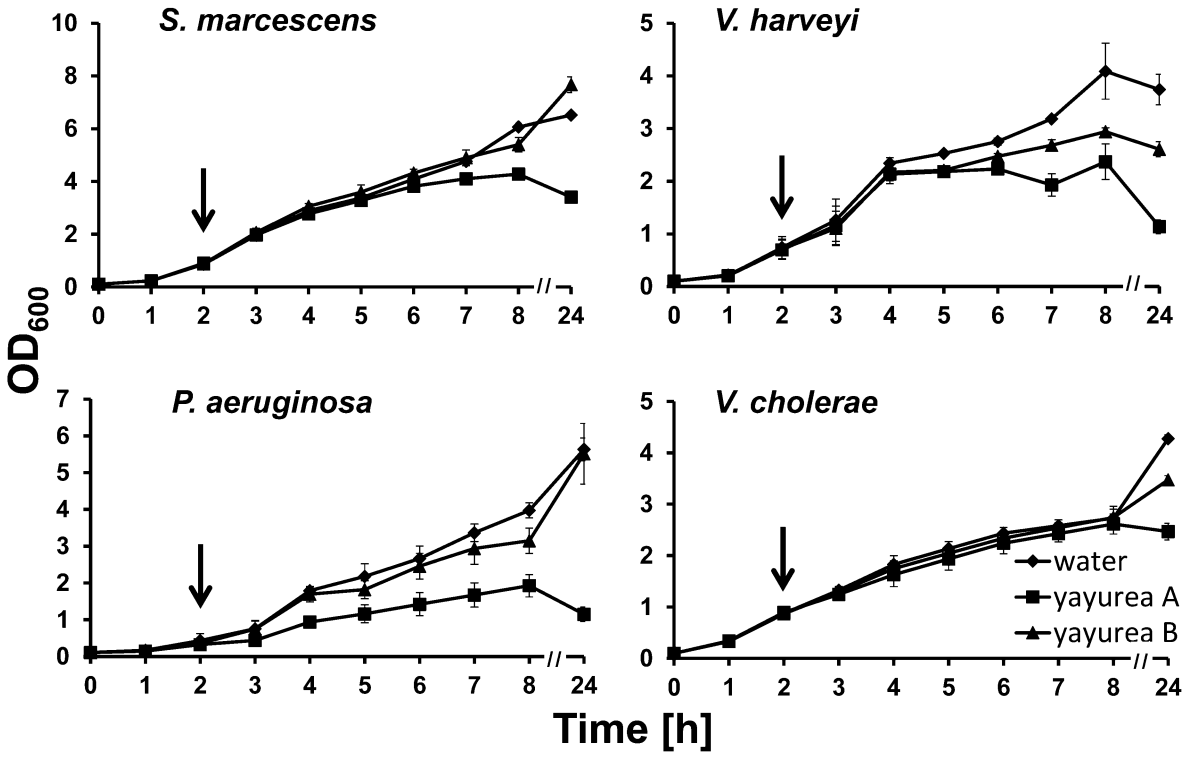


Figure 17. Influence of yayurea A and B on growth
Growth curve of *S. marcescens*, *P. aeruginosa*, *V. harveyi* and *V. cholerae* in BM with 1,000 µg/ml yayurea A or B, or an equal volume of water. Arrows indicate time points (after 2h) of compound addition to the growing cultures. All the measurements were made in triplicate. Bars indicate standard deviation, SD.

e) Yayurea A and B are perceived by the AHL-receptor LuxN of Vibrio harveyi

To gain insight into the molecular mechanisms underlying the decrease in bioluminescence of *V. harveyi* caused by yayurea A and B, we performed *in vitro* phosphorylation assays of the signaling proteins involved. The full-length hybrid kinases LuxN, LuxQ, CqsS (tagged with 6 histidine residues) were heterologously expressed in an *E. coli* strain that lacks the F_1/F_0-ATPase (to prevent ATP degradation during phosphorylation experiments), and inverted membrane vesicles prepared from this strain were directly used for the phosphorylation experiments. Then we tested the effect of yayurea A and B on the LuxN, LuxQ [in interplay with LuxP (LuxPQ)], and CqsS-mediated time-dependent phosphorylation of the HPt protein LuxU **(Fig. 18A)**. Yayurea A and B had no effect on LuxPQ or CqsS-mediated phosphorylation of LuxU **(Fig. 18B)**. However, they significantly stimulated

the LuxN-mediated phosphorylation of LuxU in comparison to the control **(Fig. 18C, compare lane 2 with lanes 4 and 10)**. LuxN is the sensor for the AHL autoinducer *N*-(3-hydroxybutyryl)-homoserine lactone (HAI-1) **(Fig. 18A)**. It is known that the presence of HAI-1 inhibits the autophosphorylation activity of LuxN, thus decreasing the level of phospho-LuxU **(Fig. 18C, compare lanes 2 and 6)**.

Yayurea A and B caused the opposite effect, leading to an increase in the level of phospho-LuxU, which explains the decrease in bioluminescence when *V. harveyi* is exposed to these compounds. Under our test conditions, autoinducer HAI-1 was dominant in relation to yayurea A or B **(Fig. 18C, compare lane 4 with lanes 8 and 12)**. Taken together, these data show that the receptor kinase LuxN of *V. harveyi* specifically recognizes yayurea A and B. It is still unclear whether yayurea A and B also bind to the HAI-1 binding site, or whether LuxN contains an independent binding site for these novel compounds.

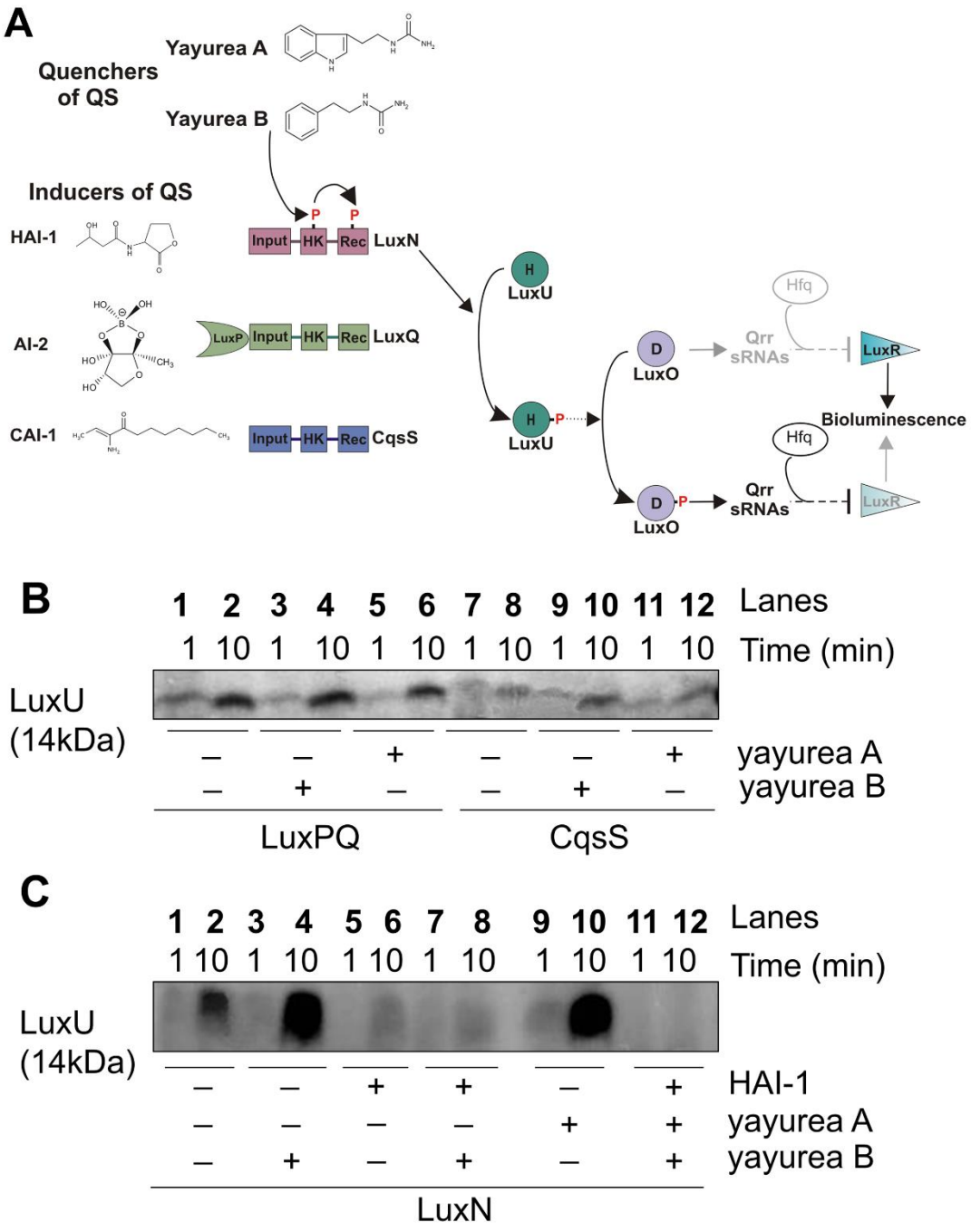


Figure 18. Yayurea A and B affect the *V. harveyi* LuxN receptor

(A) Schematic representation of the QS phosphorelay in *V. harveyi.* In the absence of autoinducers (HAI-1, AI-2 and CAI-1) at low cell density, each of the three receptors, LuxN, LuxQ and CqsS, respectively autophosphorylates at a conserved histidine of their histidine kinase domain (HK). The phosphoryl group is first transferred to the receiver domain (Rec) of the receptor kinase and then to the HPt protein LuxU. LuxP is a periplasmic binding protein. P denotes phosphorylation sites. Upon perception of the autoinducers at high cell density, autophosphorylation of the receptors and the subsequent phosphosphrylation cascade is inhibited. Yayurea A and B stimulated the phosphorylation of the cascade via LuxN. (**B**) LuxPQ and CqsS

mediated phosphorylation of LuxU in the presence of yayurea A and B. (**C**) LuxN mediated phosphorylation of LuxU in the presence of yayurea A and B and HAI-1. LuxQ, CqsS, LuxN-bearing membrane vesicles and LuxU, were incubated with 100 μM [γ-^{32}P] ATP. The effect of yayurea A, B, and HAI-1 (**C**) on the initial rate of LuxU phosphorylation was evaluated. Each reaction was sampled and stopped at two different time points: after 1 and 10 minutes. Final concentrations were 20 μM for HAI-1, 1.1 mM for yayurea A and 1.3 mM yayurea B, which were reflective of the *in vivo* situation. Absence of HAI-1 or yayurea A or B is indicated by "−", while their presence is indicated by "+".

f) Phylogenetic position of yayurea-producing species in the Staphylococcus taxa

We tested a number of staphylococcal species via co-cultivation with *P. aeruginosa* and assessment of pyocyanin production suppression **(Table 4)**. Only five species exerted such an activity: *S. delphini*, *S. intermedius*, *S. lutrae, S. pseudintermedius*, and *S. schleiferi,* and they all produced yayurea A and B, as determined by HPLC analysis. Based on 16S rRNA- and multilocus-sequence typing (MLST) the species were determined to be phylogenetically related, and are summarized in the 'intermedius group' (Lamers et al., 2012) **(Fig. 19)**. Typically, the species are coagulase-positive (with the exception of *S. schleiferi* subsp. *schleiferi*), and oxidase-negative. Interestingly, they all colonize various animals, and many represent zoonotic pathogens (Bannoehr et al., 2007; Ben Zakour et al., 2012). None of the other listed staphylococcal species (not even the closely-related *S. hyicus*, *S. chromogenes* or *S. muscae*) inhibited QS systems in Gram-negative bacteria, nor did they produce yayurea A and B.

Table 4. Strains tested for QS-quenching activity and yayurea A and B production

Strains	QS-inhibition
S. aureus RN4220	−
S. aureus SA113	−
S. aureus 8325-4	−
S. aureus RN1	−
S. aureus HG001	−
S. aureus HG002	−
S. aureus HG003	−
S. aureus Newman	−
S. arlettae DSM 20672T	−
S. carnosus TM300 DSMZ 20501	−
S. capitis subsp. *capitis* LK499 ATCC 27840	−
S. caprae DSM 20608T	−
S. chromogenes DSM 20454T	−
S. cohnii subsp *cohnii* DSM20260	−
S. condiment DSM 11674T	−
S. delphini DSM 20771	+++
S. epidermidis ATCC14990	−
S. equorum subsp. *equorum* DSM 20674T	−
S. gallinarum DSM 20610T	−
S. muscae DSM 7068T	−
S. haemolyticus CCM2737	−
S. hominis DSM 20328	−
S. hyicus NCTC 10350	−
S. intermedius CCM 5739	+
S. lentus DSM 20352T	−
S. lugdunesis ATCC 43809	−
S. lutrae DSM 10244T	+
S. pasteuri ATCC 51129	−
S. pseudintermedius ED99	+++
S. saprophyticus subsp. *saprophyticus* DSM 200229	−
S. schleiferi subsp. *coagulans* ATCC49545	++
S. schleiferi subsp. *schleiferi* DSM 4807	+++
S. simulans MK148 ATCC 27848	−
S. warneri DSM 20316T	−
S. xylosus DSM 20266	−

(+), Species that produce yayurea A and B

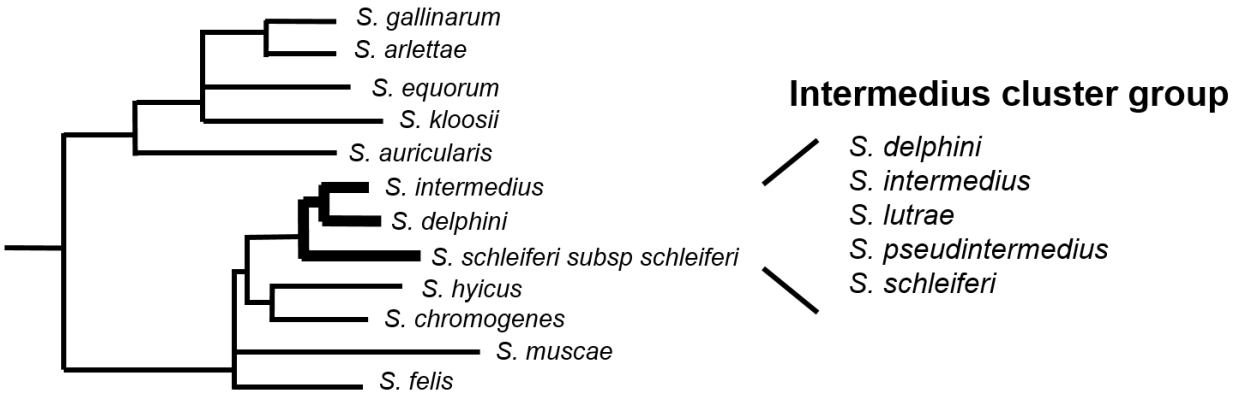


Figure 19. Phylogenetic tree of *Staphylococcus* species
The tree is based on 16S rRNA relationships according to (Götz et al., 2006). The phylogenetic position of the 'intermedius group', composed of *S. intermedius*, *S. pseudintermedius*, and *S. delphini*, is marked in bold. Based on a combination of 16S rRNA and multilocus data, the group was recently complemented by the next-of-kin species *S. lutrae*, *S. schleiferi* subsp. *schleiferi*, and *S. schleiferi* subsp. *coagulans* (Lamers et al., 2012). All members of this group produce yayurea A and B.

3. Corresponding genes of yayureas

To search the candidate genes involved in yayurea production, random transposon mutagenesis was performed with Tn917 delivery plasmid (pTV1ts) to construct pools of transposon mutants, each containing a unique insertion in *S. schleiferi subsp. schleiferi*. Supernatants of individual mutants were collected and their ability of repressing prodigiosin production in *S. marcescens* was tested. The colonies which lost prodigiosin-repressing activity were selected and analyzed for their transposon insertion site with LI-COR DNA sequencer **(Table. 5)**. Four of the mutant clones had transposon insertions interrupting known *Staphylococcus* global regulatory loci, three of which were in the *agr* (accessory gene regulation) system (twice at *agrC*) and one of which was in the *sar* (staphylococcus accessory regulation). Additional three mutant clones had transposon insertions in transporter proteins, such as sodium alanine symporter, glutamate ABC transporter, and major facilitator family transporter, secondary carriers facilitating movement of small solutes in response to chemiosmotic ion gradients (Pao et al., 1998; Walmsley et al., 1998). Four other mutants had transposon insertions in known genes of transposase for ISSps1, metallo-β-lactamase superfamily protein, glycine dehydrogenase subunit 1, and PIN domain-containing membrane protein, the last of which had a nuclease enzyme function that could cleave single-strand RNA in prokaryotes (Arcus et al., 2011). Two

clones had insertions at unknown-function putative proteins. The genes which were only shown once in the pool were not likely the corresponding genes for yayurea. We believe *agr*- and *sar* system play an important role for biosynthesis of yayurea, especially the *agr* system. However, both systems regulate numbers of downstream genes which involved in extracellular toxins, cell surface proteins, enzymes and signaling pathway. Thus we have to extent the amount of mutants in this pool and screen more transposon mutants to demonstrate connection between *agr* system and yayureas.

Table 5. Characterization of candidate clones

Transposon insertion site	**Function**
agrA (accessory gene regulator A)	response regulator of agr regulator system
agrC (accessory gene regulator c)	histidine protein kinase
sarR (accessory regulator R)	transcriptional regulator
SACOL1392	sodium alanine symporter
glutamine-binding periplasmic protein	glutamate ABC transporter
SPSE1456	major facilitator family transporter
SPSE0435	Transposase for ISSps1
SPSE2262	PIN domain-containing membrane protein
SPSE0028	metallo-β-lactamase superfamily protein
gcvPA	glycine dehydrogenase subunit 1
SPSE1358	putative membrane protein
SPSE1216	unknown function protein

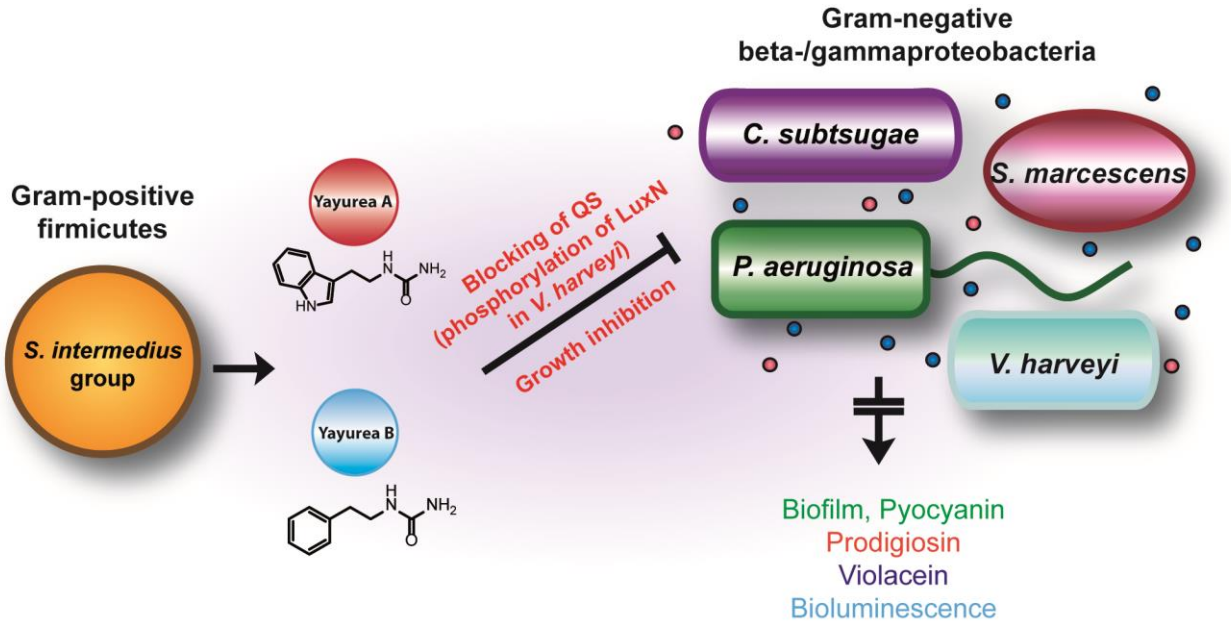


Figure 20. Schematic presentation of the interference between zoonotic staphylococcal species and Gram-negative bacteria

Animal associated (zoonotic) *Staphylococcus* species excrete novel quorum-quenching compounds, yayurea A and B, which block quorum sensing systems in various Gram-negative bacteria by activating LuxN phosphorylation, and also have growth-inhibiting activity. The benefit for staphylococcal species is better survival and increased competitiveness in a joint ecosystem.

Discussion

We found that some staphylococcal species excrete novel compounds, yayurea A and B, which interfere with the QS system of diverse Gram-negative bacteria. We tested 24 staphylococcal species with respect to production of yayurea A and B, via co-cultivation with Gram-negative bacteria. The results showed only five species (*S. delphini* (Varaldo et al., 1988), *S. intermedius* (Hájek, 1976)*, S. pseudintermedius* (van Duijkeren et al., 2011)*, S. lutrae* (Foster et al., 1997), and *S. schleiferi* (Bes et al., 2002)) produced these compounds and were able to inhibit QS-regulated markers of Gram-negative bacteria. *S. delphini* was isolated from dolphins, camels, pigeons, and *mustelidae* (minks, ferrets and badgers), mostly in association with infection (Guardabassi et al., 2012; Sledge et al., 2010). *S. pseudintermedius* has been mainly isolated from dogs, but also from other animals (Devriese et al., 2009; Devriese et al., 2005). *S. intermedius* is prevalent in domestic pigeons and dogs (Cree and Noble, 1995; Hájek, 1976; Phillips and Kloos, 1981). *S. lutrae* was classified after isolation form otters (Foster et al., 1997). *S. schleiferi* was first described as a clinical isolate, but has been isolated from the external auditory meatus of dogs with external ear otitis (Bes et al., 2002; Freney et al., 1988; Igimi et al., 1990). All five species are naturally associated with various animals, where they act as zoonotic pathogens. They are only rarely associated with human infections. It is remarkable that these species were found to be clustered in various phylogenetic trees, based on either 16S rRNA (Götz et al., 2006; Takahashi et al., 1999), thermonuclease (*nuc*) sequences (Sasaki et al., 2010), major autolysin (atl) sequences (Albrecht et al., 2012), or multilocus sequence typing (MLST) (Lamers et al., 2012). The five species form a phylogenetic cluster, termed the "intermedius group" **(Figure 19)**.

The structures of yayureas and preliminary results suggest that they are derived respectively from phenylalanine and tryptophan **(Fig. 12)**. Staphylococci are auxotrophic for a few amino acids; however, they are prototrophic for the aromatic amino acids (Rudin et al., 1974). There are two possibilities of how yayureas are synthesized: either the biosynthesis enzymes are part of the Trp- and Phe-biosynthesis pathway, or Trp and Phe are first synthesized and then modified, perhaps involving a carbamoyl transferase reaction. Yayureas have been chemically

synthesized (Wöfle *et al.* unpublished). The structures of the synthetic compounds were identical to the naturally-produced compounds, with both groups showing the same activity **(Figs. 10 and 11)**.

The first described quorum-sensing system was in the bioluminescent marine bacterium *Vibrio fischeri* (Nealson and Hastings, 1979). Later, it was observed in *Vibrio harveyi* that bacteria can communicate through multiple quorum-sensing signals, and *V. harveyi* became one of the QS model bacteria. The *V. harveyi* quorum-sensing system consists of three autoinducers and three cognate receptors, functioning in parallel to channel information into a shared regulatory pathway (Waters and Bassler, 2005). Similar to other Gram-negative bacteria, *V. harveyi* produces an AHL signal termed AI-1 (3OHC4-homoserine lactone) (Hanzelka et al., 1999), which binds to the membrane-bound sensor histidine kinase (LuxN). The second molecule is AI-2, a furanosyl borate diester, which binds to the periplasmic protein LuxP and the LuxP-AI-2 complex to interact with another membrane-bound sensor histidine kinase, LuxQ. The third molecule is termed CAI-1 (cholera autoinducer-1), an (S)-3-hydroxytridecan-4-one (Chen et al., 2002; Higgins et al., 2007), which is recognized by the membrane-bound sensor histidine kinase CqsS (Henke and Bassler, 2004). At low cell density, in the absence of appreciable amounts of autoinducers, the three sensors (LuxN, LuxQ, and CqsS) act as autophosphorylating kinases that subsequently transfer the phosphate to the cytoplasmic protein LuxU, which passes the phosphate to the DNA-binding response regulator protein LuxO (Freeman and Bassler, 1999a, b). Phospho-LuxO, in conjunction with a transcription factor termed σ^{54}, activates expression of five regulatory small RNAs (sRNAs) termed Qrr1–5 (Quorum Regulatory RNA) (Lenz et al., 2004; Lilley and Bassler, 2000), which indirectly repress the master regulator of quorum sensing, LuxR. Together, these communication systems act as a three-way coincidence detectors in the regulation of a variety of genes, including those responsible for bioluminescence, biofilm formation, type III secretion, or metalloprotease production (Henke and Bassler, 2004). To gain insight into the mode of QS quenching by yayurea A and B, we performed *in vitro* phosphorylation assays with the autoinducer receptors LuxN, LuxQ, CqsS **(Fig. 18A)**. Yayurea A and B had no effect on LuxPQ or CqsS-mediated phosphorylation of LuxU. However, they significantly stimulated the LuxN-mediated phosphorylation of LuxU **(Fig. 18B -**

C), suggesting that they interact with LuxN and induce LuxU activation. While the LuxN autoinducer HAI-1, an AHL, inhibits the autophosphorylation of LuxN, consequently leading to a decrease in LuxU-phosphorylation, yayurea A and B induced the opposite effect by increasing LuxU phosphorylation. This suggests that yayurea A and B keep *V. harveyi* in a phenotypic state of low cell density, even though the cells have grown to high density in actuality. When both AI-1 and yayurea A or B were applied, HAI-1 overruled the effect of yayurea A and B *in vitro*. Bioluminescence of the wild type strain *in vivo* significantly decreased after exposure to yayurea A and B, although the effect of the latter compound was smaller. Obviously, HAI-1 did not overrule the effect of yayurea A and B *in vivo*. This difference can be explained by the fact that bioluminescence was measured in stationary phase cells. a time when the impact of HAI-1 on QS induction is low (Anetzberger et al., 2012). Furthermore, the contribution of the other receptors (LuxQ and CqsS) and their cognate autoinducers (AI-2 and CAI-1) needs to be considered *in vivo* (Anetzberger et al., 2012). The ratio of the three receptors and the impact of their kinase and phosphatase activities on the output of the phosphorylation cascade is not yet fully understood. This could be another explanation for the strong effect of the quorum quenchers *in vivo*. Membrane-topology analysis predicts that LuxN is bound to the bacterial inner-membrane by nine transmembrane (TM) spanning domains (Jung et al., 2007), and periplasmic loop 3 might be the HAI-1 binding site (Swem et al., 2008). We believe that yayurea A and B interfere with the AHL quorum sensing response of Gram-negative bacteria; since the tested bacteria on which the effects were observed have at least one AHL-based quorum sensing system (*Pseudomonas*, *Chromobacterium*, *Vibrio*, *and Serratia*).

The *in vitro* phosphorylation assay provided initial mechanistic insights on how yayurea A and B affect quorum sensing of *V. harveyi*. We detected a significant and specific effect of yayurea A and B on the AHL-receptor LuxN. The concentrations used for these *in vitro* assays may not necessarily be similar to physiological levels, especially when the influence of both molecules (HAI-1 and yayurea) was studied. Furthermore, it should be noted that HAI-1 is not constantly produced (Anetzberger et al., 2012). Therefore, in the natural habitat there might be times when yayurea from *S. delphini* can fully interact with LuxN from *V. harveyi* in the absence of any competition with HAI-1. It therefore makes sense that the zoonotic staphylococci

impair the QS-system of Gram-negative bacteria for competitive reasons. We do not yet know whether *Vibrio harveyi* or *Serratia marcescens* excrete anti-staphylococcal compounds. However, *P. aeruginosa* excretes various respiratory toxins (e.g. pyocyanin and cyanide) that not only attack tissue cells but also inhibit growth of *S. aureus* (Lau et al., 2004; Ran et al., 2003; Voggu et al., 2006). The growth of zoonotic staphylococci is not impaired by *P. aeruginosa* because the excreted yayureas suppress the production of the QS-controlled toxins, and are thereby protected from being killed by the toxins **(Fig. 13)**. While biofilm formation appears to be modulated by many regulators and environmental conditions in *P. aeruginosa*, pyocyanin and cyanide are controlled by the Las-QS system (Rampioni et al., 2007), implying that yayurea A and B might compete with 3-oxo-C12-HSL for LasR interaction.

P. aeruginosa QS is hierarchically organized, since 3OC12-HSL is required for optimal production of the other QS signals (Heeb et al., 2011; Wilder et al., 2011). Both pyocyanin and cyanide inhibit the growth of *S. aureus* and other pathogenic staphylococci; however, pyocyanin was approximately 100 times more active than cyanide (Voggu et al., 2006). Pyocyanin, a phenazine antibiotic, is not only controlled by QS signaling (Deziel et al., 2004), but also by catabolite repression (crc), as its synthesis is 26-fold upregulated in crc mutants (Huang et al., 2012). The physiological function of pyocyanin is displayed predominantly during restricted growth and in the stationary phase (Price-Whelan et al., 2007). When yayurea A (125 µg/ml) was added, the growth of *P. aeruginosa* was not affected **(Fig. 16A)**, but pyocyanin production and biofilm formation **(Figs. 16A and 15C)** were inhibited by 20%, which indicates that biofilm inhibition takes place prior to the onset of growth inhibition. In addition to their quorum quenching activity in Gram-negative bacteria, yayureas also inhibited their growth at higher doses **(Fig. 17)**, which is a further advantage in the race for space and resources. For this advantage the zoonotic staphylococci are apparently prepared to pay a certain price, namely the production and excretion of comparatively high amounts of yayurea A and B. However, the benefit in competitiveness appears to prevail over the cost disadvantages. As QS controls not only virulence factors but also many metabolic functions important for fitness, we do not know whether the inhibition of growth is a consequence of QS-inhibition or vice versa (Dandekar et al., 2012; Heurlier et al., 2006; van Kessel et al.,

2013). For some antibiotics (azithromycin, ceftazidime, and ciprofloxacin), it has been shown that they decrease the expression of QS-regulated virulence, as well as many other genes (Skindersoe et al., 2008). The yayureas are potential candidates for use as anti-infectives. The only disadvantage might be the rather high concentration needed to completely quench QS; on the other hand, preliminary results suggest that cytotoxic effects are minimal **(Fig. 18)**.

Most natural environments harbor a stunningly diverse collection of microbial species. There are not many Gram-positive bacteria able to produce secondary metabolites that inhibit quorum sensing-controlled phenotypes in various Gram-negative bacteria. One example is the marine bacterium *Halobacillus salinus*, which produces *N*-(2'-phenylethyl)-isobutyramide and 2,3-methyl-*N*-(2'-phenylethyl)-butyramide (Teasdale et al., 2009). These compounds are similar but unrelated to yayurea A (N-[2-(*1H*-indol-3-yl)ethyl]-urea) and B (N-(2-phenethyl)-urea). Within these communities, bacteria compete with their neighbors for space and resources (Hibbing et al., 2010). Zoonotic commensals and pathogens, including yayurea-producing staphylococcal species, use animals as a habitat. Whether these animals are also colonized by Gram-negative bacteria, other than in the gut, has barely been investigated. However, it is likely that animals bathe in puddles, lakes, and rivers, suggesting that their mucus, skin, fur, or feathers may easily encounter Gram-negative bacteria, being transient to permanent colonizers. It is also very likely that the 'intermedius group' share the habitat animal with Gram-negative bacteria. *S. delphini* was isolated from dolphins. Since both *V. harveyi* and *S. delphini* are marine bacteria and animal pathogens, it is conceivable that both bacteria share the same habitat and moreover, the same host surface. Our results demonstrated suppression of the quorum sensing-regulated bioluminescence of *V. harveyi* during co-culture with *S. delphini* **(Fig. 1)**, indicating that yayurea A and B are effective quorum quenchers.

Our study unveiled a number of interesting questions. First, what are the genes encoding the biosynthetic enzymes of yayurea A and yayurea B? The result of transposon mutagenesis suggested that yayureas are controlled by *agr* and *sar*, which are global regulatory systems in Staphylococci **(Table. 6)**. We assume that special lactone peptides in the *S. intermedius* group (SIG) might regulate certain downstream genes that are controlled in different ways in *S. aureus*, which then trigger the biosynthetic pathway of yayureas. However, we still do not know what

these downstream genes are. Second, are there compounds with better effects than yayureas? According to this result and precursor tests in **Fig. 12**, it was suggested that the quorum quenching activity of yayureas may primarily depend upon the side chain. Moreover, AHLs in different species of Gram-negative bacteria vary in the length and composition of the acyl side chain of autoinducers (Taga and Bassler, 2003), which also indicates that the acyl side chain plays an important role for biological activity. For further medical use, it is necessary to conduct more tests on yayureas and their derivatives in the future. Last but not least, why is yayurea production particular to the SIG, and what is the benefit for SIG? The most likely answer is that this group shares its habitat with Gram-negative bacteria. There must be a benefit to producing yayurea A and B because they are excreted in high amounts that certainly expend precious energy and resources. In addition, the yayureas show growth inhibition only at quantities that exceed the physiological concentrations. It is a clever arrangement, in that *S. delphini* produces just enough yayurea A and B to almost completely silence the expression of the studied QS-regulated compounds or biofilm formation in diverse Gram-negative bacteria. In mixed cultures, the yayurea-producing staphylococci arrest Gram-negative bacteria in a pheno- and genotypic state of low cell density, although the cells have grown to high densities in actuality. The advantage for the staphylococci is twofold: on the one hand, they are protected from QS-controlled toxins, and on the other hand, yayurea A and B affect stationary growth of those Gram-negative bacteria with prominent QS-control systems. This is one of the rare cases of inter-phylum interference between firmicutes (Gram-positive) and beta-/gamma-proteobacteria (Gram-negative). A schematic presentation of the interference is shown in **Fig. 20**.

Part II The role of protein A in *Staphylococcus aureus* adherence to ethylene glycol coated surfaces

Introduction

Biofilm formation is an important cause of chronic infections. The formation of biofilm involves in multiple steps, stating with a nonspecific and reversible attachment (Otto, 2008). Once the biofilm is formed, the bacteria are nearly invulnerable as they are protected from the immune system and antibiotic treatment. Thus, it is very important to prevent biofilm formation and an effective way is to avoid the initial adhesion of microorganisms to the surface.

In order to prevent microorganisms from building a biofilm, the surface should be very smooth or coated with some compounds. The widest used biofilm defense strategy on indwelling medical devices is chemical coating. Antimicrobial coatings such as antibiotics, ion- and biocide coating are often used to prevent biofilm formation(Dror et al., 2009). Another effective chemical coating are long and flexible polymeric chains which can be anchored to the surfaces by covalent bonds. Self-assembled monolayer (SAMs) based surface coatings with oligo-ethylene-glycol (OEG) or poly-ethylene-glycol (PEG) terminations render surfaces resistant against non-specific protein adsorption and bacterial adhesion, which appears to be a promising approach (Pale-Grosdemange et al., 1991; Prime and Whitesides, 1993)

In this study, we prepared OEG (EG_3OMe) and PEG (PEG2k and PEG5k) coated surfaces that had good physicochemical properties in preventing *S. aureus* adhesion (Schilp et al., 2009). Pretreatment of EG_3OMe and PEG coatings with γ-globulins or serum strongly promoted adherence of *S. aureus* when resuspended in buffer, suggesting that γ-globulins play a pivotal role in promoting *S. aureus* adhesion by its IgG binding proteins; the finding that a *spa*-deletion mutant, lacking the IgG binding protein A, showed decreased adherence corroborated this.

Results and discussion

In cooperation with the research group of Dr. Fajun Zhang (Institut für Angewandte Physik, Universität Tübingen), the EG_3OMe and PEG2k coated surfaces were tested for bacterial adhesion. The uncoated gold surface was heavily covered with *S. aureus* cells, while the EG_3OMe and PEG2k coated surfaces were hardly covered with bacteria (**Fig. 24 a, b and c**). As implant material usually comes in contact with plasma components it is important to know what influence they have on bacterial adhesion. The coated surfaces were pre-incubated with PBS, bovine serum albumin (BSA), γ-globulins and bovine serum and tested for bacterial adhesion. When the coated surfaces were pre-incubated with BSA, there was no adherence of *S. aureus*, however, if pre-incubated with γ-globulins or bovine serum, the adhesion of *S. aureus* was significantly promoted (**Fig. 24 d, e and f**). Interestingly, we noticed that the amount of attached bacteria after pre-incubating with pure γ-globulins (60 mg/ml) was six-fold higher than that after pre-incubating with serum (the γ-globulins were about 10 mg/ml) (**Fig. 24 d and e**).

The pretreatment of EG_3OMe and PEG2k coatings with γ-globulins or bovine serum caused massive adhesion of *S. aureus* cells. We assume this might be due to that *S. aureus* harbors a high immunoglobulin-binding activity caused by its cell-wall bound protein A (Spa) (Löfdahl et al., 1983; Uhlen et al., 1984). The "second immunoglobulin-binding protein" (Sbi) is secreted and should not play a major role in adherence (Zhang et al., 1998). To prove this hypothesis, *S. aureus* SA113Δ*spa* (pCX-*pp-sfgfp*), which is a Protein A deficient mutant strain, was used for the tests on uncoated gold or PEG5K coated surfaces. When the bacteria were resuspended in PBS buffer, the uncoated gold surface was heavily covered by the Wild-type (WT) and *spa* mutant strains (**Fig. 25A1 and B1**). If the strains were resuspended in buffer there was no adherence to the PEG5K coated surface (**Fig. 25A2 and B2**). When the PEG5K coated surface was pretreated with γ-globulins, the WT showed good adherence, whereas the Δ*spa* mutant did not adhere to surfaces (**Fig. 25A3 and B3**). In addition, we were interested to see if blocking Protein A would affect the bacterial adhesion. To address this question, *S. aureus* was resuspended in bovine serum and tested for its adherence on different coated surfaces. When the bacteria were resuspended in bovine serum, neither WT *S. aureus* nor Δ*spa* mutant adhered to the surfaces (**Fig. 25A4 and B4**).

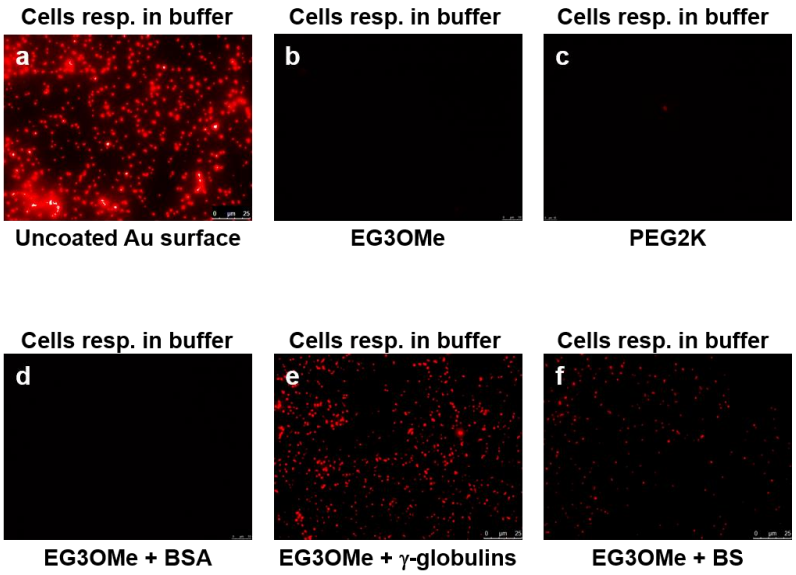


Figure 21. Effect of *S. aureus* cells resuspended in PBS buffer on adherence to EG_3OMe and PEG2k coated surfaces. Fluorescence microscopy images of *S. aureus* (pC-*tuf-ppmCh*) adhered on uncoated Au surface (a), EG_3OMe coated surface (b), PEG2k coated surface (c) and EG_3OMe coated surfaces pre-incubated with 20 mg/ml BSA (d), 50 mg/ml γ-globulins (e) or 100% bovine serum (f). (modified according to Yu *et al*. manuscript accepted)

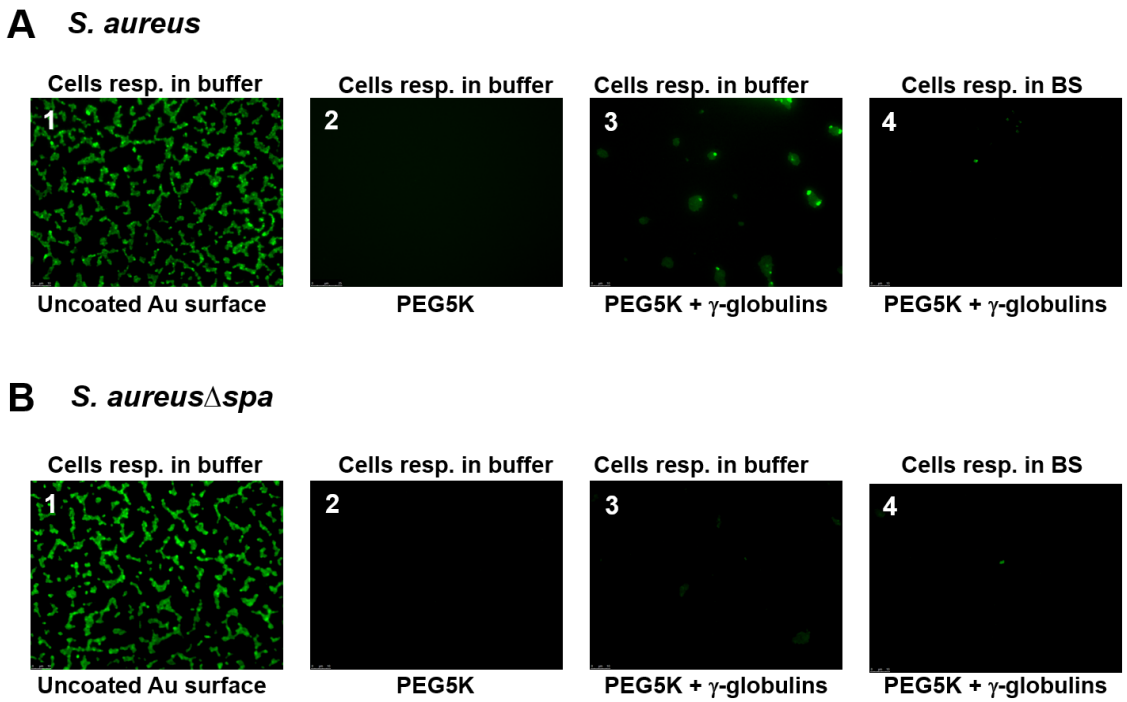


Figure 22. Comparison of *S. aureus* WT (**A**) and protein A mutant (**B**) on adherence. Fluorescence microscope images of *S. aureus* adhesion on uncoated gold (Au) surfaces (1), PEG5k coated (2) and PEG5k coated surfaces pre-incubated with 50 mg/ml γ-globulins (3). Wafers were incubated with *S. aureus* SA113 WT (pC-*tuf-gfp*) or SA113Δ*spa* (pCX-*pp-sfgfp*); cells were either resuspended in PBS (buffer) (1-3) or bovine serum (BS) (4).

Thus, our data demonstrated the adhesion of *S. aureus* cells on EG_3OMe and PEG coatings and the effects pretreating these coatings with albumin, γ-globulins and serum. When coated with EG_3OMe and PEG, the surfaces were hardly covered by bacteria (**Fig. 21**). However, pre-incubating EG_3OMe and PEG coated surfaces with γ-globulins or serum strongly promoted *S. aureus* adhesion (**Fig. 21**). Comparisons of the adhesion of *S. aureus* WT with a protein A mutant showed that protein A played a crucial role in adherence when the coatings were pretreated with serum or γ-globulins (**Fig. 22B3**). The result of resuspending *S. aureus* with bovine serum also supported this hypothesis (**Fig. 22B4**). However, the interaction between γ-globulins and the coatings is still unknown. Are there special binding domains in γ-globulins that interact with the PEG and EG_3OMe coatings? Is the Fc or the Fab part of IgG involved in mediating the binding to PEG and EG_3OMe? These questions deserve future investigation.

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List of publications

Publications from this work

Chu, Y. Y., Nega, M., Wölfle, M., Plener, L., Grond, S., Jung, K., and Götz, F. (2013) A new class of quorum quenching molecules from *Staphylococcus* species affects communication and growth of gram-negative bacteria. *PLoS pathogens* **9**, e1003654

Chu, Y. Y., Nega, M., and Götz, F. (2014) Purification and Structural Analysis of QS-inhibiting Compounds from *Staphylococcus delphini. Bio-protocol* 4(11): http://www.bio-protocol.org/wenzhang.aspx?id=1146.

Schuster, S., Yu, W., Nega, M., Chu, Y. Y., Zorn, S., Zhang, F., Götz, F. and Schreiber, F. (2014) The role of serum proteins in *Staphylococcus aureus* adhesion to ethylene glycol coated surfaces. *IJMM.*

Nollmann, F. I., Heinrich A., Brachmann, A. O., Morisseau, C., Mukherjee, K., Casanova-Torres, Á. M., Kinski, S., Schultz, K., Beeton, M., Kaiser, M., Chu, Y. Y., Ke, L. P., Thanwisai, A., Bozhüyük, K. A., Chantratita, N., Götz, F., Waterfield, N. R., Vilcinskas, A., Goodrich-Blair, H., Hammock, B. D., and Bode, H. B. (2014) A *Photorhabdus* natural product inhibits insect juvenile hormone epoxide hydrolase. Submitted to *Chembiochem.*

Publications not from this work

Lin, T. Y., Chu, Y. Y., Yang, Y. C., Hsu, S. W., Liu, S. T., and Chang, L. K. (2014) MCAF1 and Rta-activated BZLF1 transcription in Epstein-Barr virus. *PloS one* **9**, e90698

Contribution to publications

Chu, Y. Y., Nega, M., Wölfle, M., Plener, L., Grond, S., Jung, K., and Götz, F. (2013) A new class of quorum quenching molecules from *Staphylococcus* species affects communication and growth of gram-negative bacteria. *PLoS pathogens* **9**, e1003654
I planned, designed, performed and analyzed the biological activity experiments of yayurea with various methods. Also I played a part in the compounds purification procedure and structure analysis and also contributed to data analysis. The manuscript was drafted and executed by me.

Chu, Y. Y., Nega, M., and Götz, F. (2014) Purification and Structural Analysis of QS-inhibiting Compounds from *Staphylococcus delphini. Bio-protocol* 4(11): http://www.bio-protocol.org/wenzhang.aspx?id=1146.
I summed up and wrote the purification and structure analysis protocol in detail according our study.

Schuster, S., Yu, W., Nega, M., Chu, Y. Y., Zorn, S., Zhang, F., Götz, F. and Schreiber, F. (2014) The role of 1 serum proteins in *Staphylococcus aureus* adhesion to ethylene glycol coated surfaces. *IJMM* accepted.
I preformed the adhesion experiment with various mutant strains according to the experimental design and reviewer's request, and responded to the reviewer during the revision process, including rewriting part of the article, editing figures, writing the rebuttal and re-submitting the paper.

Nollmann, F. I., Heinrich A., Brachmann, A. O., Morisseau, C., Mukherjee, K., Casanova-Torres, Á. M., Kinski, S., Schultz, K., Beeton, M., Kaiser, M., Chu, Y. Y., Ke, L. P., Thanwisai, A., Bozhüyük, K. A., Chantratita, N., Götz, F., Waterfield, N. R., Vilcinskas, A., Goodrich-Blair, H., Hammock, B. D., and Bode, H. B. (2014) A *Photorhabdus* natural product inhibits insect juvenile hormone epoxide hydrolase. Submitted to *Chembiochem.*
I verified the biological activity of the phurealipids in accordance to the experimental design for their quorum sensing and growth inhibition against *P. aeruginosa* and *S. marcescens*.

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Appendix: Publications incorporated in this study

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A New Class of Quorum Quenching Molecules from *Staphylococcus* Species Affects Communication and Growth of Gram-Negative Bacteria

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1 Interfaculty Institute of Microbiology and Infectious Diseases Tübingen (IMIT), Microbial Genetics, University of Tübingen, Tübingen, Germany, **2** Organic Chemistry, University of Tübingen, Tübingen, Germany, **3** Munich Center for Integrated Protein Science (CiPSM) at the Department of Microbiology, Ludwig-Maximilians-Universität München, Martinsried, Germany

Abstract

The knowledge that many pathogens rely on cell-to-cell communication mechanisms known as quorum sensing, opens a new disease control strategy: quorum quenching. Here we report on one of the rare examples where Gram-positive bacteria, the '*Staphylococcus intermedius* group' of zoonotic pathogens, excrete two compounds in millimolar concentrations that suppress the quorum sensing signaling and inhibit the growth of a broad spectrum of Gram-negative beta- and gamma-proteobacteria. These compounds were isolated from *Staphylococcus delphini*. They represent a new class of quorum quenchers with the chemical formula *N*-[2-(*1H*-indol-3-yl)ethyl]-urea and *N*-(2-phenethyl)-urea, which we named yayurea A and B, respectively. *In vitro* studies with the N-acyl homoserine lactone (AHL) responding receptor LuxN of *V. harveyi* indicated that both compounds caused opposite effects on phosphorylation to those caused by AHL. This explains the quorum quenching activity. Staphylococcal strains producing yayurea A and B clearly benefit from an increased competitiveness in a mixed community.

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Introduction

In many bacteria, pathogenicity is controlled and coordinated by an inter-cellular communication process named quorum sensing (QS). QS is based on the synthesis and secretion of small hormone-like molecules termed autoinducers that bind to cognate receptors. The signal-activated receptor controls directly or indirectly expression of target genes. Since the concentration of signaling molecules in liquid culture is proportional to cell density in the culture, gene expression is coordinated in response to the bacterial population density [1,2]. In *V. harveyi*, the QS system consists of three autoinducers and three cognate receptors functioning in parallel to channel information into a shared regulatory pathway [2]. Similar to other Gram-negative bacteria, *V. harveyi* produces an AHL signal termed HAI-1, 3-hydroxy-C4-homoserine lactone [3], which binds to the membrane-bound sensor histidine kinase (LuxN). The second molecule is AI-2, a furanosyl borate diester, which binds to the periplasmic protein LuxP. The LuxP-AI-2 complex interacts with another membrane-bound sensor histidine kinase, LuxQ. The third molecule is termed CAI-1 (for cholera autoinducer-1), a long chain ketone [4,5], which is recognized by the membrane-bound sensor histidine kinase, CqsS [6]. At low cell density, in the absence of appreciable amounts of autoinducers, the three sensors (LuxN, LuxQ, and CqsS) act as autophosphorylating kinases that subsequently transfer the phosphate to the cytoplasmic protein LuxU, which passes the phosphate to the DNA-binding response regulator protein LuxO [7,8]. Phosphorylated LuxO represses the master regulator of QS, LuxR, via sigma factor σ^{54} and regulatory small RNAs [9,10].

Similar to *V. harveyi*, *P. aeruginosa* coordinates the expression of nearly 10% of its genome through three hierarchically arranged QS systems, namely Las, Rhl and Pqs [11]. Each system consists of enzymes involved in autoinducer synthesis and the target regulator: LasI produces 3-oxo-C12-HSL for activation of LasR [12], RhlI produces C4-HSL for the activation of RhlR [13,14], and the biosynthetic enzymes PqsABCDE and PhnAB produce PQS (2-heptyl-3-hydroxy-4-quinolone) for activation of PqsR [15–17]. QS systems are also prevalent in many other Gram-negative bacteria.

QS system is a promising target for anti-virulence therapy [1,18]. In contrast to classic antibiotics, quorum-quenching compounds are inhibitors of bacterial virulence, rather than of bacterial growth [19]. Since the first studies on QS inhibitors, the halogenated furanones [20], more compounds have been identified [21,22].

In contrast to Gram-negative bacteria, many Gram-positive bacteria communicate using modified oligopeptides as signals and

Author Summary

While studying the potential interaction of staphylococci with Gram-negative bacteria, we came across another communication system in a *Staphylococcus* species group, which consists of closely related coagulase-positive bacterial species that play a role as zoonotic pathogens. We found that these species excrete two small compounds that inhibit both the expression of QS-controlled toxins and other QS-regulated compounds as well as growth in Gram-negative bacteria. The excreted compounds, which we named yayurea A and B, were isolated from *S. delphini* and structurally characterized. They represent new bacterial products, which quench the QS regulation in a wide spectrum of Gram-negative bacteria by stimulating the LuxN-mediated phosphorylation of LuxU. Furthermore, growth of yayurea A and B producing *S. delphini* is not suppressed by respiratory toxins when co-cultured with *P. aeruginosa*. This suggests that the quorum quenchers have a function in self-protection and competitiveness in natural environments shared with Gram-negatives. Here we show one of the rare cases of inter-phylum interference between firmicutes (Gram-positive) and beta-/gammaproteobacteria (Gram-negative).

"two-component"-type membrane-bound sensor histidine kinases as receptors. The well-studied QS system in *Staphylococcus* is the agr QS system [23]. The excreted signal is a thiolactone- or lactone-based peptide [24] (AIP, autoinducer peptide) that mediates communication with other staphylococci in a cell density dependent way [25,26].

While studying the potential interaction of staphylococci with Gram-negative bacteria [27,28], we came across another communication system in a *Staphylococcus* species group, named 'intermedius group'. This group consists of closely related mainly coagulase-positive bacterial species including *S. delphini*, *S. intermedius*, *S. lutrae*, *S. pseudintermedius*, and *S. schleiferi*. They are all phylogenetically related, are zoonotic pathogens, and only rarely occur in human infections. We found that these species excrete two low molecular compounds that inhibit the expression of QS-controlled toxins and other QS-regulated compounds in Gram-negative bacteria. The excreted compounds, which we named yayurea A and B, were isolated from *S. delphini* and structurally characterized. Yayurea A and B represent new bacterial products, and were able to quench the QS regulation in a wide spectrum of Gram-negative bacteria. Furthermore, growth of yayurea A and B producing *S. delphini* is not suppressed by respiratory toxins when co-cultured with *P. aeruginosa*. This suggests that the quorum quenchers have a function in self-protection and competitiveness in natural environments shared with Gram-negatives. Here we show an example of inter-phylum interference between Firmicutes (Gram-positive) and the Gram-negative beta- and gammaproteobacteria.

Results

Staphylococcus delphini suppresses production of QS-regulated phenotypes in various Gram-negative bacteria

Our aim was to find out if some staphylococcal species are able to suppress the QS controlled phenotypes in Gram-negative bacteria. To investigate this, we tested the ability of several staphylococcal species to inhibit pyocyanin production of *P. aeruginosa* in a co-cultivation assay, as pyocyanin production is QS controlled. We found that *S. delphini* DSMZ 20771 completely inhibited pyocyanin production over 24 h co-cultivation with *P. aeruginosa* PAO1, while *Staphylococcus aureus* showed no such activity (Fig. 1A). This led us to investigate if *S. delphini* could also suppress QS-controlled phenotypes in other Gram-negative bacteria such as the QS-regulated prodigiosin production in *Serratia marcescens* [29]; bioluminescence in *Vibrio harveyi* [7,30]; or violacein production in *Chromobacterium subtsugae*, a pathogen of potato beetles [31]. Indeed, in co-cultivation studies with these Gram-negative bacteria, *S. delphini* did also suppress prodigiosin and violacein production as well as bioluminescence, while *S. aureus* did not (Fig. 1BCD). Sterile filtered culture supernatant of a 24 h *S. delphini* culture had the same QS-inhibiting effect as the co-culture, indicating that the QS-inhibiting compound(s) were excreted. The supernatants of *S. aureus* and *S. epidermidis* caused no effect.

Structural analysis of the QS-inhibiting compounds from *S. delphini*

The QS inhibitors were isolated from the supernatant of an overnight culture of *S. delphini* DSMZ 20771. Further purification revealed that the supernatant contained two compounds with different retention times (R_t) in HPLC and distinct UV spectra. We named the two compounds yayurea A and B.

Yayurea A (indole-ethylurea) was isolated as a brownish solid, revealed an ion peak at $m/z = 161$ ($[M+H]^+$) in ESI-MS, and showed the molecular formula, $C_{10}H_{13}N_2$, in FT-ICR-MS analysis. The 1*H*-indole-3-ethylamine moiety was deduced from GC-EI-MS ($R_t = 29.0$ min). ^{1}H-NMR spectra showed a characteristic singlet at $\delta_H = 8.55$ ppm. The remaining signals were assigned to 2-(3-indoyl) ethylamine. The ^{13}C-NMR spectrum and additional HSQC experiments displayed signals pointing to a carbonyl group at $\delta = 170.4$ ppm, five methin groups, and two methylene groups to reveal a urea moiety. Additionally, 2D NMR experiments supported the structure of yayurea A as *N*-[2-(1H-indole-3-yl) ethyl]-urea (Fig. 2A).

Yayurea B (phenethylurea) was obtained as a colorless solid. A preliminary molecular formula, $C_8H_{10}N$, was deduced from the FT-ICR-MS spectrum which showed an ion at $m/z = 121$ ($[M+H]^+$). GC-EI-MS provided a signal at $R_t = 15.0$ min pointing to 2-phenethylamine. The ^{1}H-NMR again showed a set of aromatic protons and a singlet at $\delta_H = 8.54$ ppm, while the ^{13}C-NMR displayed a signal at $\delta_H = 170.4$ ppm (C = O) and five aromatic and 4 aliphatic protons. In summary, the structure of *N*-(2-phenethyl)-urea was assigned for yayurea B (Fig. 2B). UV-Absorption maxima emphasized the phenyl- ($\lambda = 260$ nm) and indole chromophores ($\lambda = 225$ nm and $\lambda = 280$ nm respectively) of the yayureas. Comprehensive physicochemical characteristics and mass spectra of yayurea A and B are shown (Table 1 and Figure S1). In the meantime, yayurea A and B could also be chemically synthesized and their identity confirmed by mass spectral and NMR analyses (Wölfle *et al.* manuscript in preparation). Both the synthesized and the natural compounds revealed the same chemical properties and the same QS-quenching activity in Gram-negative bacteria (data not shown).

Suppression of QS-regulated respiratory toxins protects *S. delphini* from killing by *Pseudomonas aeruginosa*

P. aeruginosa produces various QS-controlled respiratory toxins such as pyocyanin and hydrogen cyanide [32,33], which kill *S. aureus* [27]. If yayurea A and B repress the production of QS-regulated pyocyanin or hydrogen cyanide, one would expect that *S. delphini* survives better in a co-culture with *P. aeruginosa* than, for example, *S. aureus*. Indeed, co-

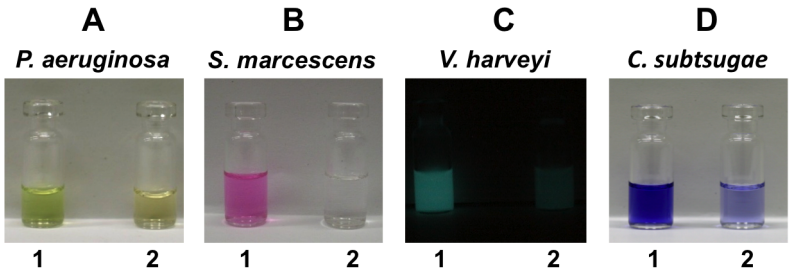

Figure 1. Quenching of QS-regulated pigments and bioluminescence by *S. delphini*. *P. aeruginosa* (**A**), *S. marcescens* (**B**), *V. harveyi* (**C**) and *C. subtsugae* (**D**) were each co-cultivated with *S. aureus* (1) or *S. delphini* (2) for 24 h. Pyocyanin, which is excreted by *P. aeruginosa*, was determined in the supernatant at its absorption maximum $A_{520\ nm}$. Prodigiosin, which is cell wall bound in *S. marcescens*, was ethanol-extracted from the cell pellet and determined at its absorption maximum $A_{534\ nm}$. Bioluminescence of *V. harveyi* was intensified by aeration before measuring in a bioluminescence reader. Violacein from *C. subtsugae* was quantitatively extracted with butanol and determined at its absorption maximum $A_{585\ nm}$.
doi:10.1371/journal.ppat.1003654.g001

cultivation studies with *S. aureus* (non-producer) or *S. delphini* with *P. aeruginosa* SH1 revealed that the viability (CFU) of *S. aureus* significantly decreased in the stationary growth phase, most likely due to the respiratory toxins produced by *P. aeruginosa* (Fig. 3A), while that of *S. delphini* was uninfluenced (Fig. 3B). The addition of yayurea A and B to the mixed *S. aureus* and *P. aeruginosa* culture protected *S. aureus* from

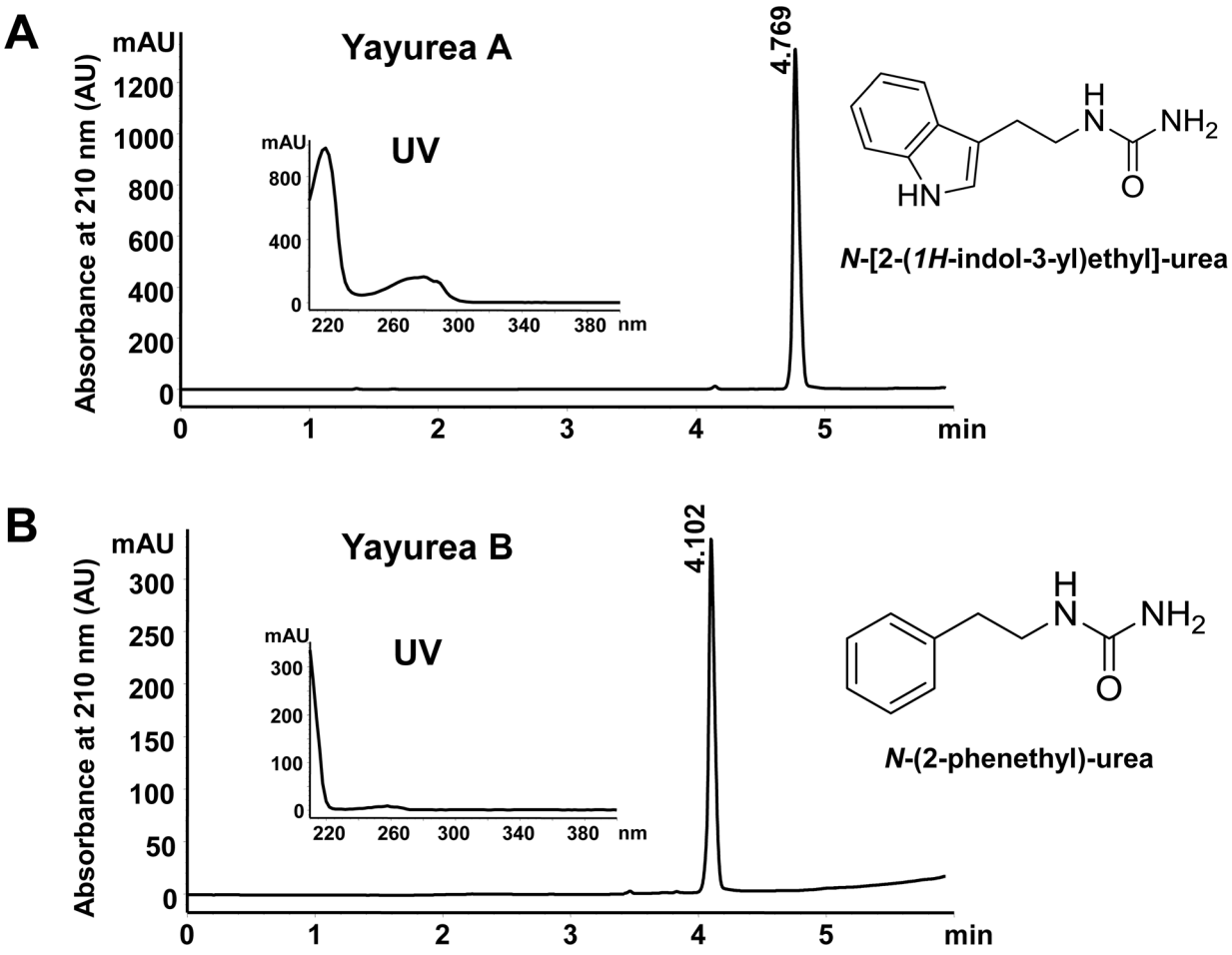

Figure 2. RP-HPLC profile, UV-spectrum and structures of the two QS-inhibitors purified from *S. delphini*. (**A**) QS-inhibitor, *N*-[2-(1H-indol-3-yl)ethyl]-urea (yayurea A). (**B**) QS-inhibitor, *N*-(2-phenethyl)-urea (yayurea B). RP-HPLC was carried out on an Agilent 1200 and Waters xBridge C18, 5 mm, 4.6×150 mm column; compounds were eluted with a 15 min linear gradient of 0.1% phosphoric acid to acetonitrile at a flow rate of 1.5 ml/min.
doi:10.1371/journal.ppat.1003654.g002

Table 1. Physical data for yayurea A and B.

	N-[2-(*1H*-indol-3-yl)ethyl]-urea (yayurea A)	*N*-(2-phenethyl)-urea (yayurea B)
Formula	$C_{11}H_{13}O_1N_3$ (203.24 g/mol)	$C_9H_{11}O_1N_2$ (163.20 g/mol)
Melting point	240.8°C	214.9°C
R_f-values	0.10 ($CHCl_3$/MeOH 9:1) 0.63 (MeOH/H_2O 7:3)	0.28 ($CHCl_3$/MeOH 9:1) 0.94 (MeOH/H_2O 7:3)
^{1}H NMR	(600 MHz, MeOH-d_4) d 3.11 (t, J = 7.0, 7.4 Hz, 2H), 3.22 (t, J = 7.1, 7.3 Hz, 2H), 7.04 (dd, J = 7.4, 7.5 Hz, 1H), 7.13 (dd, J = 7.7, 7.9 Hz, 1H), 7.17 (s, 1H), 7.37 (d, J = 8.2 Hz, 1H), 7.57 (d, J = 7.9 Hz, 1H), 8.55 (s, 1H).	(600 MHz, MeOH-d_4) d 2.95 (t, J = 7.4, 8.1 Hz, 2H), 3.16 (t, J = 7.4, 8.1 Hz, 2H), 7.28 (m, 3H), 7.35 (m, 2H), 8.54 (s, 1H).
^{13}C NMR	(150 MHz, MeOH-d_4) d 24.9, 41.5, 110.6, 112.7, 119.0, 120.2, 122.9, 124.4, 128.4, 138.5, 170.5.	(150 MHz, MeOH-d_4) d 34.9, 42.1, 128.4, 129.9, 130.2, 138.1, 170.2.
MS (ESI)	(Positive ions) *m/z* (%) $[M+2H\text{-}CONH_2]^+$ 161.11.	(Positive ions) *m/z* (%) $[M+2H\text{-}CONH_2]^+$ 122.10.

doi:10.1371/journal.ppat.1003654.t001

killing in the stationary phase (Fig. 3A). Furthermore, the CFU of *P. aeruginosa* SH1 was unaffected while co-cultured with *S. delphini* or *S. aureus*, indicating that none of the two staphylococcal species was able to kill *P. aeruginosa*. Co-cultivation of *P. aeruginosa* with *S. aureus* in the presence of yayureas (100 μg/ml yayurea A and 900 μg/ml yayurea B) had also no effect on viability of *P. aeruginosa* (Fig. 3C). Other tested *Pseudomonas* strains PAO1 and DSMZ 50071 showed similar results (data not shown). All in all, these results showed that production of yayurea A and B enables staphylococci to coexist with Gram-negative bacteria in a mixed community.

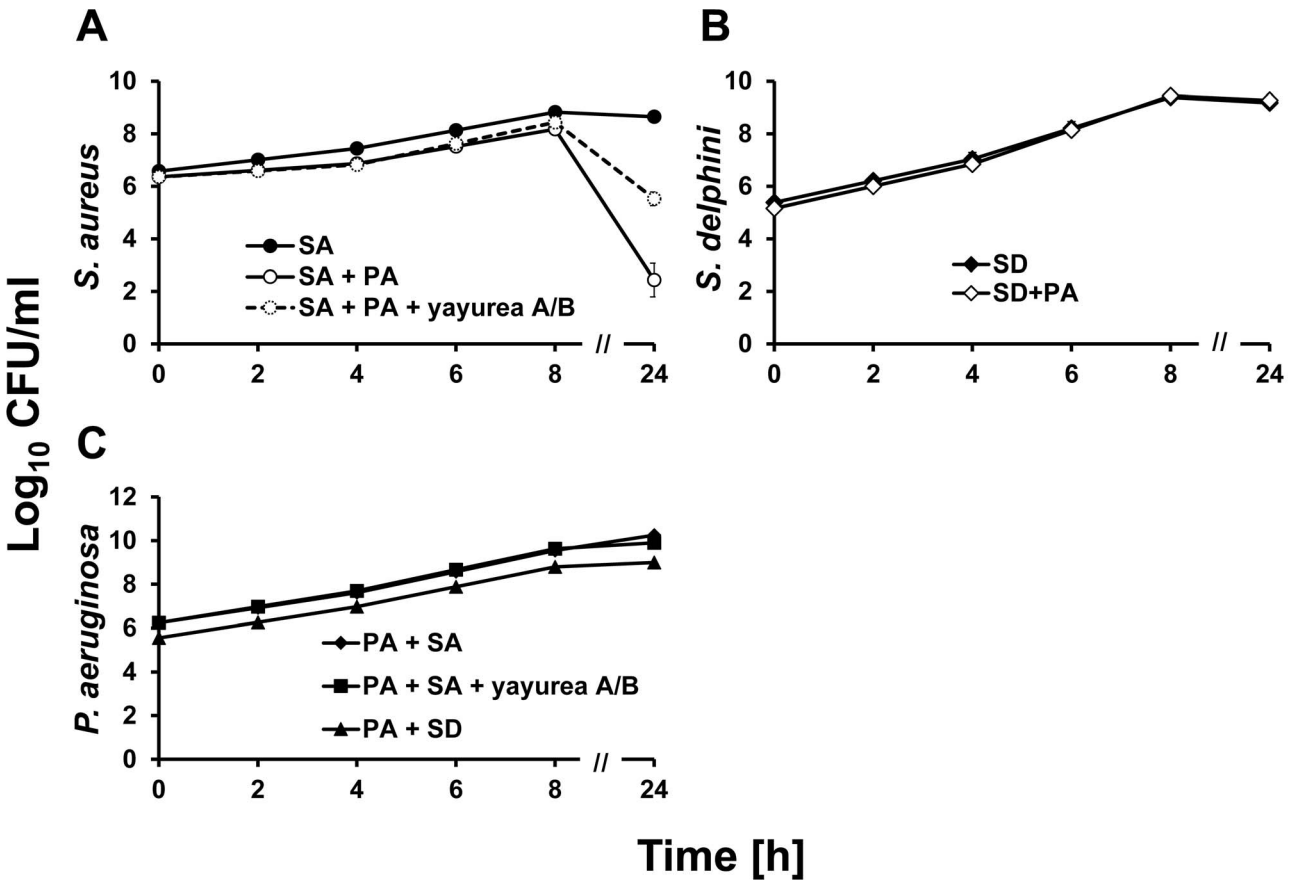

Figure 3. Survival of staphylococcal strains in mixed culture with *P. aeruginosa*. (**A**) CFU of *S. aureus* alone (SA) and in co-culture with *P. aeruginosa* (SA+PA). For the protection test, yayurea A (100 μg/ml) and B (900 μg/ml) were added to the mixture of *S. aureus* and *P. aeruginosa* (SA+PA+yayurea A/B). (**B**) CFU of *S. delphini* alone (SD) and in co-culture with *P. aeruginosa* (SD+PA). (**C**) CFU of *P. aeruginosa* SH1 co-cultured with *S. aureus* (PA+SA), yayureas (PA+SA+yayurea A/B) or *S. delphini* (PA+SD). Values represent the means of three independent experiments. Bars indicate mean standard deviation, SD.
doi:10.1371/journal.ppat.1003654.g003

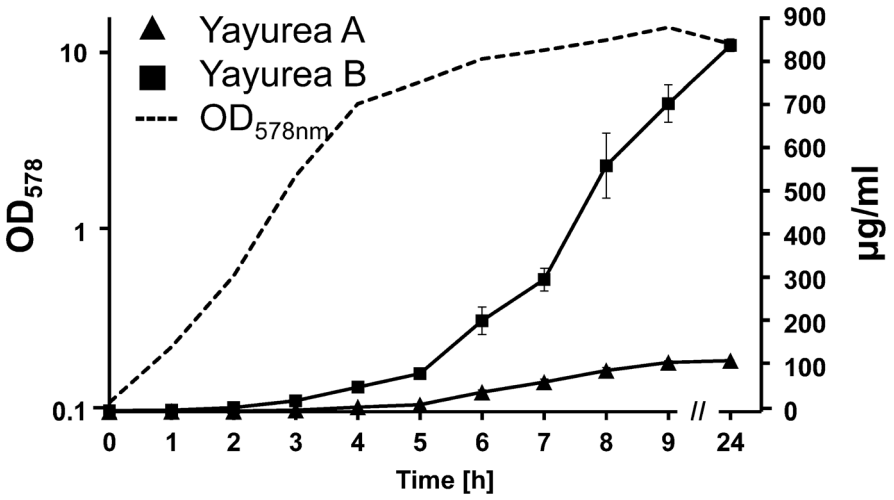

Figure 4. Quantification of yayurea A and B production in relation to growth. *S. delphini* was grown in TSB at 37°C. Supernatant was collected and OD_{578} was measured hourly for the first 9 h and after 24 h. Amounts of yayurea A and B in supernatants were quantified by triplicate HPLC measurements. Bars indicate mean standard deviation, SD.
doi:10.1371/journal.ppat.1003654.g004

Yayurea A and B are mainly produced in stationary growth phase of *S. delphini*

We followed the production of yayurea A and B over 24 h in the supernatant *S. delphini* grown in TSB. The amount of produced yayurea A and B was determined by HPLC-analysis; peak integration was correlated with standard yayurea A and B. The production of both compounds started at the transition of exponential to stationary growth phase (after approximately 4 h) and increased rapidly for the next 5 h; after 24 h little more was produced (Fig. 4). The production kinetics is reminiscent of a QS controlled expression. Both compounds were produced in amazingly high concentrations: yayurea A reached concentration of 120 µg/ml and yayurea B even 900 µg/ml. This high concentration is entirely sufficient to suppress QS-systems in Gram-negative bacteria as can be seen below.

Yayurea A is more active than yayurea B in inhibiting various QS-controlled traits in Gram-negative bacteria

Purified yayurea A and B inhibited QS-regulated factors in Gram-negative bacteria in a dose-dependent manner. We tested prodigiosin production in *S. marcescens*, bioluminescence in *V. harveyi*, and pyocyanin production in *P. aeruginosa* (Fig. 5 and 6).

Yayurea A and B inhibited production of prodigiosin in *S. marcescens* in a dose-dependent way. Inhibition already started at low concentrations (15 µg/ml) and increased with increasing concentrations of yayurea A or B. At 250 µg/ml, prodigiosin production was inhibited at 60% (yayurea A) and 40% (yayurea B). At a concentration of 1000 µg/ml, prodigiosin production was completely inhibited by yayurea A, and to approximately 70% by yayurea B (Fig. 5A). At 500 µg/ml, bioluminescence *of V. harveyi* was inhibited by yayurea A and B by 99% and 76% respectively (Fig. 5B). We also investigated whether yayurea A and B inhibited biofilm formation in *P. aeruginosa* and *S. aureus*. Again yayurea A quite efficiently inhibited biofilm formation in *P. aeruginosa*, while yayurea B was less effective (Fig. 5C). In contrast to gallidermin, a good biofilm inhibitor in staphylococci [34], both yayureas showed no biofilm-inhibiting effect with *S. aureus* (Fig. 5D).

We noticed that high dose of yayurea A and B (especially yayurea A) inhibit the growth of Gram-negative bacteria. We used *P. aeruginosa* as an example to verify the effect of growth and QS inhibition by yayurea A and B, and used the antibiotic tetracycline and the well-known QS-inhibitor furanone [35] as controls (Fig. 6). For QS inhibition, around 50% of the yayurea A and B concentration was necessary compared to that needed for growth inhibition (Fig. 6 A, B). Furanone revealed a similar correlation of growth and QS inhibition; the concentration (31 µg/ml) that inhibited 40% of growth inhibited 80% of QS (Fig. 6C). In contrast to yayurea and furanone, tetracycline inhibited growth and QS almost linearly (Fig. 6D). Besides *P. aeruginosa*, yayureas affect also growth of *S. marcescens*, *V. harveyi*, and *V. cholerae* (Figure S2); however, growth of *E. coli* was not affected. All staphylococcal species tested, such as *S. aureus*, *S. carnosus*, *S. delphini* or *S. schleiferi*, are resistant to yayurea A and B, independently whether they are producing these compounds or not.

Yayurea A and B are perceived by the AHL-receptor LuxN of *Vibrio harveyi*

To gain insight into the molecular mechanism behind why yayurea A and B cause a decrease in bioluminescence of *V. harveyi*, we performed *in vitro* phosphorylation assays of the corresponding signaling proteins. The full-length hybrid kinases LuxN, LuxQ, CqsS (tagged with 6 histidine residues) were heterologously expressed in an *E. coli* strain that lacks the F_1/F_o-ATPase (to prevent ATP degradation during phosphorylation experiments), and inverted membrane vesicles prepared from this strain were directly used for the phosphorylation experiments. Then we tested the effect of yayurea A and B on the LuxN, LuxQ [in interplay with LuxP (LuxPQ)], and CqsS-mediated time-dependent phosphorylation of the HPt protein LuxU (Fig. 7A). Yayurea A and B had no effect on LuxPQ or CqsS-mediated phosphorylation of LuxU (Fig. 7B). However, they significantly stimulated the LuxN-mediated phosphorylation of LuxU in comparison to the control (Fig. 7C, compare lane 2 with lanes 4 and 10). LuxN is the sensor for the AHL autoinducer *N*-(3-hydroxybutyryl)-homoserine lactone (HAI-1) (Fig. 7A). It is known [36] that the presence of HAI-1 inhibits the autophosphorylation activity of LuxN, thus decreasing the level of phospho-LuxU (Fig. 7C, compare lanes 2 and 6).

Yayurea A and B caused the opposite effect, leading to an increase in the level of phospho-LuxU, which explains the

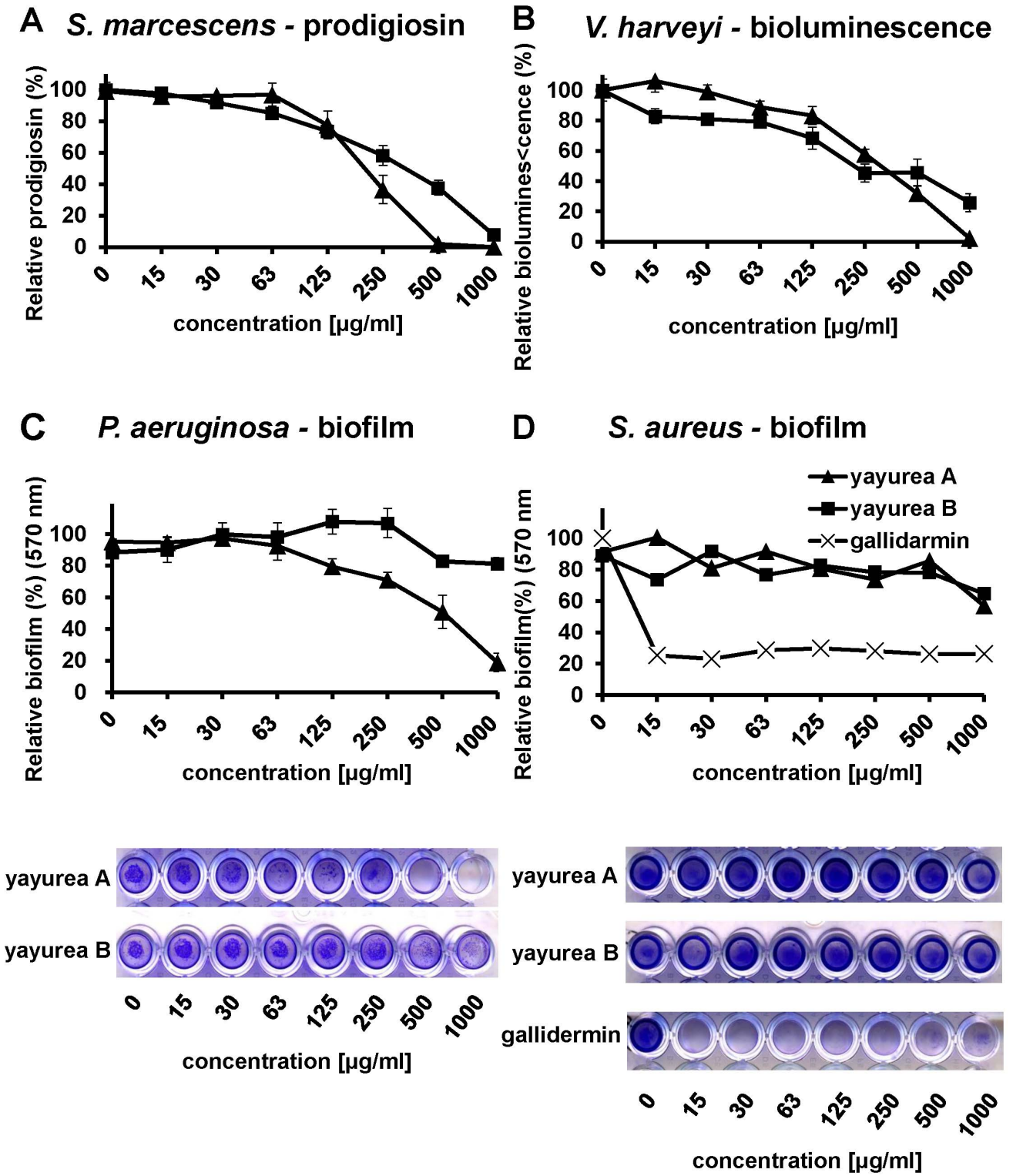

Figure 5. Concentration-dependent inhibition of QS-regulated phenotypes in Gram-negative bacteria. (**A**) Prodigiosin production in *S. marcescens*. Cells were grown in MB medium with serial dilutions of yayurea A or B at 28°C. Relative prodigiosin production was calculated as the ratio between prodigiosin content (absorbance at 534 nm) and cell density (absorbance at 600 nm). (**B**) Bioluminescence in *V. harveyi*. Cells were grown in marine broth with serial dilutions of the compounds at 28°C for 24 h. Relative luminescence units were normalized by the cell density. (**C**) Biofilm formation of *P. aeruginosa*. Cells were grown in LB with serial dilutions of yayurea A or B at 37°C for 24 h. (**D**) Biofilm formation of *S. aureus*. Cells were grown in TSB with serial dilutions of yayurea A, B, or gallidermin (positive control) at 37°C for 24 h. Biofilm cell layer was visualized by crystal violet staining and measured at 590 nm. Microtiter plates presented are representative of at least three independent sets of experiments. Bars indicate standard deviation of the mean, SD.
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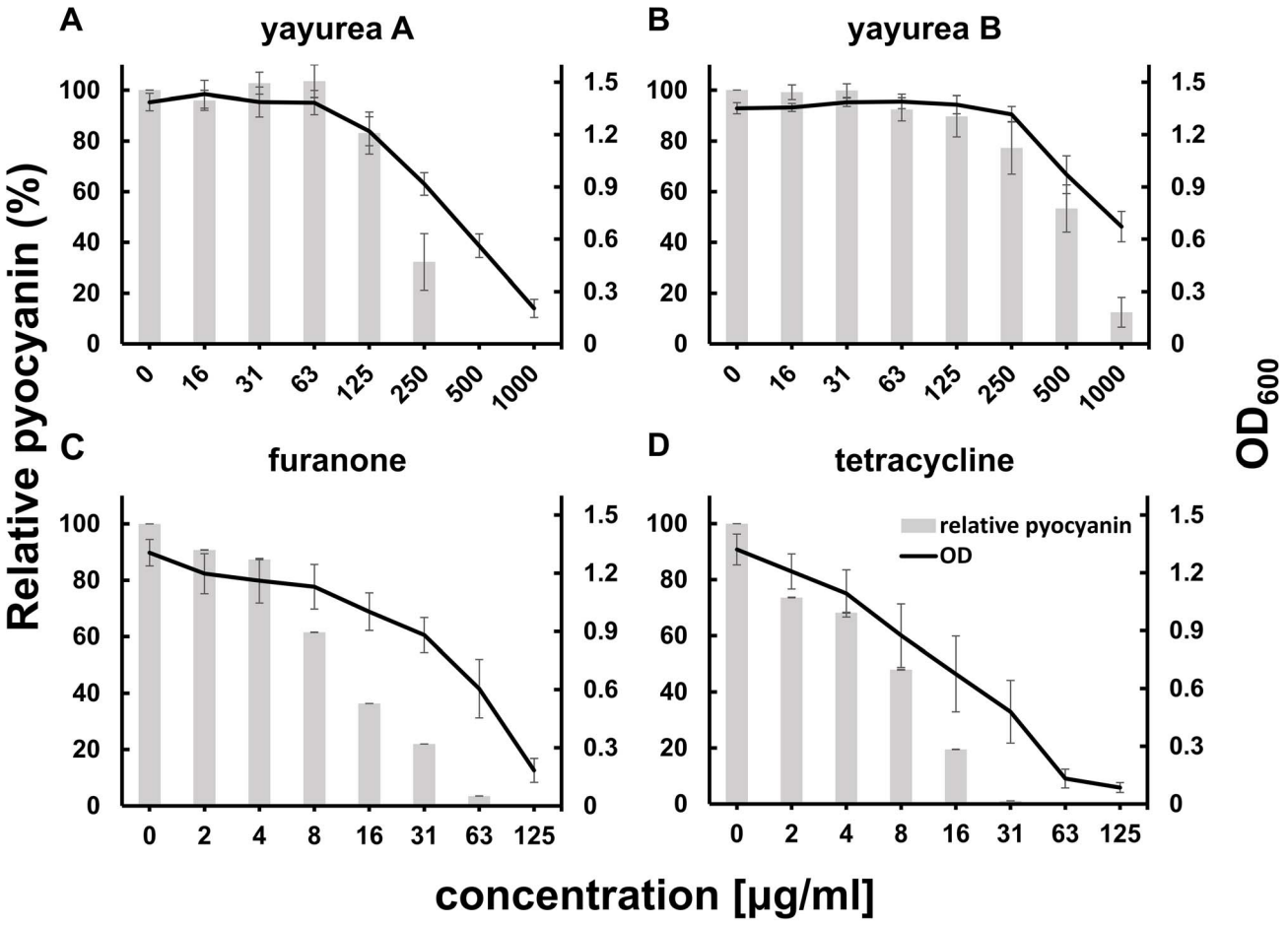

Figure 6. Concentration-dependent inhibition of pyocyanin production and growth of *P. aeruginosa*. *P. aeruginosa* PAO1 was grown in LB at 30°C with serial dilutions of yayurea A (**A**), yayurea B (**B**), furanone (**C**) and tetracycline (**D**). Relative pyocyanin production was calculated as the ratio between pyocyanin content and cell density (absorbance at 600 nm). Values represent the means of three independent experiments. Bars indicate standard deviation of the mean, SD.
doi:10.1371/journal.ppat.1003654.g006

decrease in bioluminescence when *V. harveyi* is exposed to these compounds. Under our test conditions, autoinducer HAI-1 was dominant in relation to yayurea A or B (Fig. 7C, compare lane 4 with lanes 8 and 12). Taken together these data show that the receptor kinase LuxN of *V. harveyi* specifically recognizes yayurea A and B. It is still unclear whether yayurea A and B also bind to the HAI-1 binding site or whether LuxN contains an independent binding site for these new compounds.

Phylogenetic position of yayurea A and B producing species in the *Staphylococcus* taxa

We tested a number of staphylococcal species representatives (listed in Table 2) by co-cultivation with *P. aeruginosa* for suppression of pyocyanin production. Only five species exerted such an activity: *S. delphini*, *S. intermedius*, *S. lutrae*, *S. pseudintermedius*, and *S. schleiferi*, and they all produced yayurea A and B as determined by HPLC analysis. Based on 16S rRNA- and *multilocus sequence typing* (MLST) the species are phylogenetically related and summarized in the 'intermedius group' [37] (Figure S3). Typically, the species are coagulase positive (with exception of *S. schleiferi* subsp. *schleiferi*), and oxidase negative. Interestingly, they all colonize various animals, and many represent zoonotic pathogens [38,39]. None of the other listed staphylococcal species (even not the next related *S. hyicus*, *S. chromogenes* or *S. muscae*) inhibited QS-system in Gram-negative bacteria or produced yayurea A and B.

Discussion

In this study, we found that some staphylococcal species excrete two novel compounds, yayurea A and B that interfere with the QS system of diverse Gram-negative bacteria. We tested 24 staphylococcal species with respect to production of yayurea A and B by co-cultivation with Gram-negative bacteria. The results showed that only five species (*S. delphini* [40], *S. intermedius* [41], *S. pseudintermedius* [42], *S. lutrae* [43], and *S. schleiferi* [44]) produced these compounds and were able to inhibit QS-regulated markers of Gram-negative test bacteria. All five species are naturally associated with various animals, where they act as zoonotic pathogens. They are only rarely associated with human infections. It is remarkable that these species are clustered in various phylogenetic trees, based on either 16S rRNA [45,46], on thermonuclease (*nuc*) sequences [47], on major autolysin (atl) sequences [48], or on multilocus sequence typing (MLST) [37]. The five species form a phylogenetic cluster, termed "intermedius group" (Figure S3).

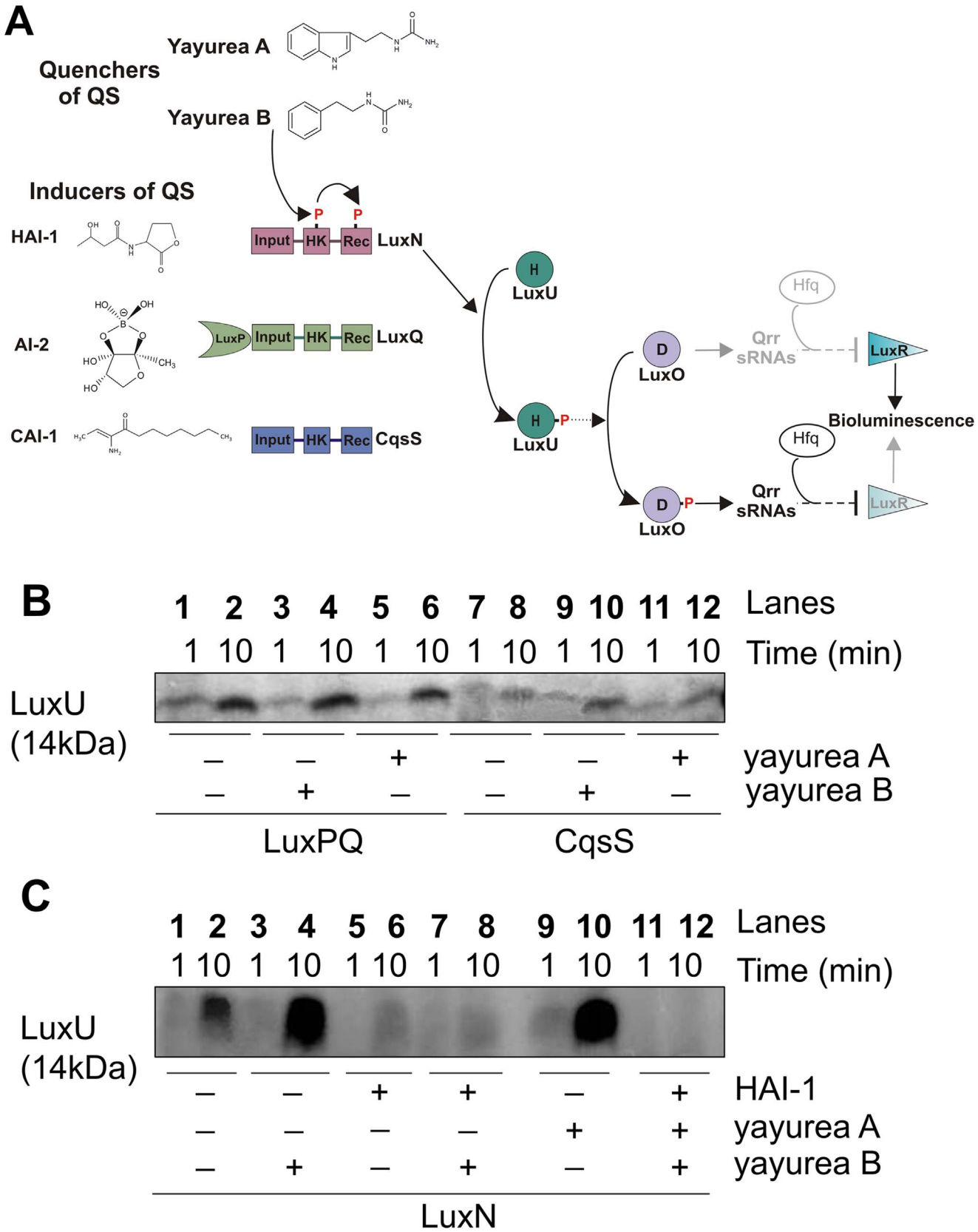

Figure 7. Yayurea A and B are perceived by *V. harveyi* LuxN receptor. (**A**) Schematic representation of the QS phosphorelay in *V. harveyi*. In the absence of autoinducers (HAI-1, AI-2 and CAI-1) at low cell density, each of the three receptors, LuxN, LuxQ and CqsS, respectively, autophosphorylates at a conserved histidine of their histidine kinase domain (HK). The phosphoryl group is first transferred to the receiver domain (Rec) of the receptor kinase and then to the HPt protein LuxU. LuxP is a periplasmic binding protein. P denotes phosphorylation sites. Upon

perception of the autoinducers at high cell density, autophosphorylation of the receptors and the subsequent phosphosphrylation cascade is inhibited. Yayurea A and B stimulated the phosphorylation of the cascade via LuxN. (**B**) LuxPQ and CqsS mediated phosphorylation of LuxU in the presence of yayurea A and B. (**C**) LuxN mediated phosphorylation of LuxU in the presence of yayurea A and B and HAI-1. LuxQ, CqsS, LuxN-bearing membrane vesicles and LuxU, were incubated with 100 μM [γ-^{32}P] ATP. The effect of yayurea A, B and HAI-1 (**C**) on the initial rate of LuxU phosphorylation was evaluated. Each reaction was sampled and stopped at two different time points: after 1 and 10 minutes. Final concentrations were 20 μM for HAI-1, 1.1 mM for yayurea A and 1.3 mM yayurea B, which reflects the *in vivo* situation. Absence of HAI-1 or yayurea A or B is indicated by "−" and presence by "+".
doi:10.1371/journal.ppat.1003654.g007

To gain insight into the mode of QS quenching by yayurea A and B, we performed *in vitro* phosphorylation assays with the autoinducer receptors LuxN, LuxQ, CqsS (Fig. 7A). Yayurea A and B had no effect on LuxPQ or CqsS-mediated phosphorylation of LuxU. However, they significantly stimulated the LuxN-mediated phosphorylation of LuxU (Fig. 7B–C), suggesting that they interact with LuxN and cause LuxU activation. While the LuxN autoinducer HAI-1, an AHL, inhibits the autophosphorylation of LuxN, consequently leading to a decrease in LuxU-phosphorylation, yayurea A and B caused the opposite effect by increasing LuxU phosphorylation. Thus, it is suggested that yayurea A and B keep the *V. harveyi* in a phenotypic state of low cell density, although the cells have grown to high density.

Table 2. Strains tested for Q-quenching activity and yayurea A and B production.

Strains	QS-inhibition
S. aureus RN4220	−
S. aureus SA113	−
S. aureus 8325-4	−
S. aureus RN1	−
S. aureus HG001	−
S. aureus HG002	−
S. aureus HG003	−
S. aureus Newman	−
S. arlettae DSMZ 20672T	−
*S. carnosus*TM300 DSMZ 20501	−
S. capitis subsp. *capitis* LK499 ATCC 27840	−
S. caprae DSMZ 20608T	−
S. chromogenes DSMZ 20454T	−
S. cohnii subsp. *cohnii* DSMZ20260	−
S. condiment DSMZ 11674T	−
S. delphini DSMZ 20771	+++
S. epidermis ATCC14990	−
S. equorum subsp. *equorum* DSMZ 20674T	−
S. gallinarum DSMZ 20610T	−
S. muscae DSMZ 7068T	−
S. haemolyticus CCM2737	−
S. hominis DSMZ 20328	−
S. hyicus NCTC 10350	−
S. intermedius CCM 5739	+
S. lentus DSMZ 20352T	−
S. lugdunesis ATCC 43809	−
S. lutrae DSMZ10244T	+
S. pasteuri ATCC 51129	−
S. pseudintermedius ED99	+++
S. saprophyticus subsp. *saprophyticus* DSMZ 200229	−
S. schleiferi subsp. *coagulans* ATCC49545	++
S. schleiferi subsp. *schleiferi* DSMZ 4807	+++
S. simulans MK148 ATCC 27848	−
S. warneri DSMZ 20316T	−
S. xylosus DSMZ 20266	−

(+), Species that produce yayurea A and B.
doi:10.1371/journal.ppat.1003654.t002

When both AI-1 and yayurea A or B were applied, HAI-1 overruled the effect of yayurea A and B *in vitro*. Bioluminescence of the wild type strain *in vivo* significantly decreased after exposure to yayurea A and B, although the effect of the latter compound was smaller. Obviously, HAI-1 did not overrule the effect of yayurea A and B *in vivo*. This difference can be explained by the fact that bioluminescence was measured in stationary phase grown cells at a time when the impact of HAI-1 on QS induction is low [49]. Furthermore, the contribution of the other receptors (LuxQ and CqsS) and their cognate autoinducers (AI-2 and CAI-1) needs to be considered *in vivo* [49]. The ratio of the three receptors and the impact of their kinase and phosphatase activities on the output of the phosphorylation cascade is not yet fully understood. This could be another explanation for the strong effect of the quorum quenchers *in vivo*. Membrane-topology analysis predicts that LuxN is bound to the bacterial inner-membrane by nine transmembrane (TM) spanning domains [50], and periplasmic loop 3 might be the HAI-1 binding site [21]. We believe that yayurea A and B interfere with the AHL quorum sensing response of Gram-negative bacteria; since the tested bacteria on which the effects were observed have at least one AHL-based quorum sensing system (*Pseudomonas*, *Chromobacterium*, *Vibrio*, *and Serratia*).

Most natural environments harbor a stunningly diverse collection of microbial species. One example, is the marine bacterium *Halobacillus salinus*, which produces *N*-(2′-phenylethyl)-isobutyramide and 2,3-methyl-*N*-(2′-phenylethyl)-butyramide [51]. These compounds are unrelated to yayurea A (N-[2-(*1H*-indol-3-yl)ethyl]-urea) and B (N-(2-phenethyl)-urea). Within these communities, bacteria compete with their neighbors for space and resources [52]. Zoonotic commensals and pathogens, including yayurea producing staphylococcal species, use animals as a habitat. Whether these animals are also colonized by Gram-negative bacteria, other than in the gut, has barely been investigated. However, one can assume that animals bathe in puddles, lakes, and rivers, suggesting that their mucus, skin, fur, or feathers may easily encounter Gram-negative bacteria, being transient to permanent colonizers. One can also assume, that the 'intermedius group' share the habitat animal with Gram-negative bacteria. *S. delphini* was isolated from dolphin. Since both *V. harveyi* and *S. delphini* are marine bacteria and animal pathogens, it is conceivable that both bacteria share the same habitat and moreover, the same host surface. Our results demonstrated suppression of the quorum sensing regulated bioluminescence of *V. harveyi* during co-culture with *S. delphini* (Fig. 1), indicating that yayurea A and B are effective quorum quenchers.

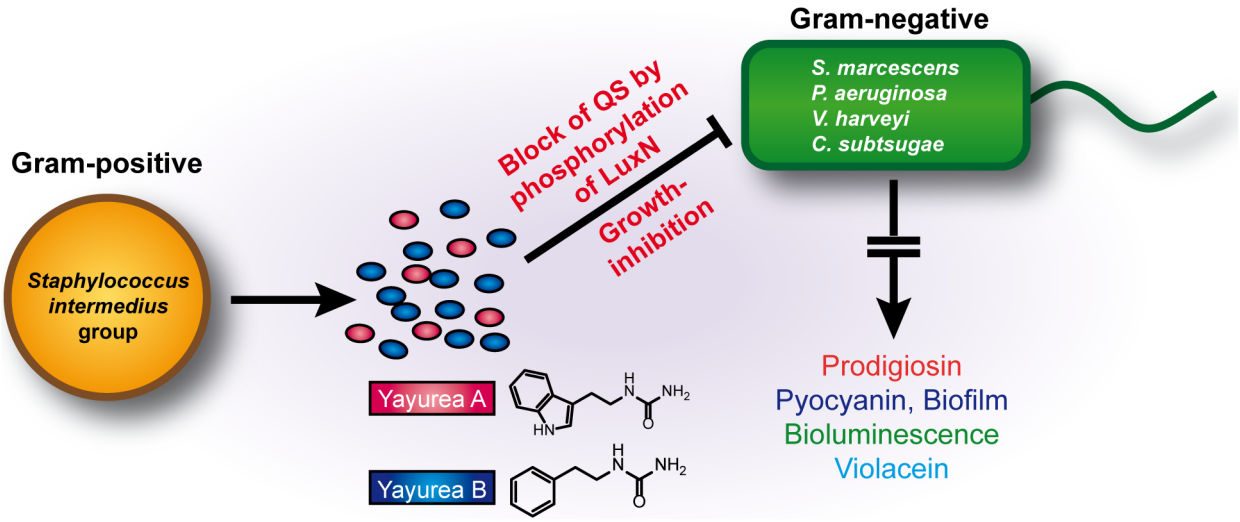

Figure 8. Schematic presentation of the interference between zoonotic staphylococcal species and Gram-negative bacteria. Animal associated (zoonotic) *Staphylococcus* species excrete novel quorum-quenching compounds, yayurea A and B, which block quorum sensing system in various Gram-negative bacteria by activating LuxN phosphorylation and have also growth inhibiting activity. The benefit for the staphylococcal species is better survival and increased competitiveness in a joint ecosystem.
doi:10.1371/journal.ppat.1003654.g008

The *in vitro* phosphorylation assay gave first mechanistic insights on how yayurea A and B affect quorum sensing of *V. harveyi*. We detected a significant and specific effect of yayurea A and B on the AHL-receptor LuxN. The concentrations used for these *in vitro* assays might not be physiological, especially when the influence of both molecules (HAI-1 and yayurea) was studied. Furthermore, it should be noted that HAI-1 is not constantly produced [49]. Therefore, in the natural habitat there might be times, when yayurea from *S. delphini* can fully interact with LuxN from *V. harveyi* in the absence of any competition with HAI-1. It therefore makes sense that the zoonotic staphylococci impair the QS-system of Gram-negative bacteria for competitive reasons. The growth of these staphylococci is not impaired by *P. aeruginosa* because the excreted yayureas suppress the production of the QS-controlled toxins and are thereby protected from being killed by the toxins (Fig. 3). While biofilm formation appears to be modulated by many regulators and environmental conditions in *P. aeruginosa*, pyocyanin and cyanide are controlled by the Las-QS system [53], implying that yayurea A and B might compete with 3-oxo-C12-HSL for LasR interaction.

When yayurea A (125 µg/ml) was added, the growth of *P. aeruginosa* was not affected (Fig. 6A), but quorum sensing and biofilm formation (Figs. 6A and 5C) were inhibited by 20%, which indicates that biofilm inhibition takes place prior to the onset of growth inhibition. In addition to their quorum quenching activity in Gram-negative bacteria, yayureas also inhibited their growth at higher dose (Fig. 2), which is a further advantage in the race for space and resources. For this advantage the zoonotic staphylococci are apparently prepared to pay a certain price, namely the production and excretion of comparatively high amounts of yayurea A and B. However, the benefit in competitiveness appears to prevail the cost disadvantages. As QS controls not only virulence factors but also many metabolic functions important for fitness, we don't know whether the inhibition of growth is a consequence of QS-inhibition or vice versa [54–56]. For some antibiotics (azithromycin, ceftazidime, and ciprofloxacin), it has been shown that they decrease the expression of QS-regulated virulence and many other genes [57].

The yayureas are potential candidates for use as anti-infectives. The only disadvantage might be the rather high concentration needed to completely quench QS; on the other hand, preliminary results suggest that they hardly have cytotoxic activities.

An interesting question is why just the 'intermedius group' is producing yayureas. The most likely answer is that this group shares its habitat with Gram-negative bacteria. There must be a benefit to producing yayurea A and B because they are excreted in such high amounts certainly costing energy. In addition, the yayureas show growth inhibition only at quantities that exceed the physiological concentrations. It is a clever arrangement, that *S. delphini* produces just enough yayurea A and B to almost completely silence the expression of the studied QS-regulated compounds or biofilm formation in diverse Gram-negative bacteria. In mixed cultures, the yayurea-producing staphylococci arrest Gram-negative bacteria in a pheno- and genotypic state of low cell density, although the cells have grown to high density. The advantage for the staphylococci is twofold, on one hand, they are protected from QS-controlled toxins and on the other hand, yayurea A and B affect stationary growth of particularly those Gram-negative bacteria with prominent QS-control systems. This is one of the rare cases of inter-phylum interference between firmicutes (Gram-positive) and beta-/gammaproteobacteria (Gram-negative). A schematic presentation of the interference is shown in Fig. 8.

Materials and Methods

Bacterial strains and growth conditions

Staphylococcus strains were grown in Tryptic Soy Broth (TSB, Sigma) medium at 37°C, *Vibrio cholerae* SP27459 (O1 El Tor), *Serratia marcescens*, *Chromobacterium subtsugae* DSMZ 17043 and *Pseudomonas aeruginosa* were grown in lysogenic broth (LB) medium, and *Vibrio harveyi* DSMZ6904 in marine broth (MB; 5 g peptone, 3 g yeast extract and 75% sea water per liter of deionized water) at 28°C. The *Staphylococcus* strains tested in this work are listed in Table 2.

Mixed cultivation of *Staphylococcus sp.* with *P. aeruginosa*

In co-culture experiments, we inoculated TSB with *S. aureus* (OD_{578} 0.005) and *P. aeruginosa* SH1 (OD_{578} 0.005). For the protection test, yayurea A (100 µg/ml), yayurea B (900 µg/ml), or water (control) were added to the mixed culture of *S. aureus* and *P. aeruginosa* after 3 h incubation. For co-cultivation of *S. delphini* with *P. aeruginosa*, each of the strains was inoculated with OD_{578} 0.001, because doubling time of *S. delphini* was shorter than that of *S. aureus*. Co-cultures were aerobically grown at 37°C. For CFU determination, samples were diluted and plated on Chapman agar (selective medium for staphylococci, while growth of *P. aeruginosa* is retarded) and BM agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% K_2HPO_4, 0.1% glucose) on which *P. aeruginosa* grows better than the staphylococci. Colonies were counted after incubating for 24 h at 37°C; staphylococcal and pseudomonas colonies could be easily distinguished by colony shape and pigmentation.

Purification and structural analysis of the QS-inhibiting compounds from *S. delphini*

The QS inhibitors were isolated from aerobically-grown overnight culture of *S. delphini* (vigorous shaking at 37°C, 20 h). Cells were centrifuged and the supernatant was applied to an Amberilite XAD 16 resin (Rhom& Haas, Germany). The column was first washed with water, then with 40% and 60% methanol and finally eluted with solvent A (80% methanol containing 5% acetic acid). Yayureas were significantly enriched in the last fraction. In the second purification step, the eluate was evaporated *in vacuo*, suspended in water, and further separated on an Amberilite IRC 50 cation exchange resin (Serva, Heidelberg, Germany) after the pH was adjusted to 7.0 with 1 M NaOH. The column was washed with water, then 70% ethanol and eluted with solvent A. The eluate was concentrated *in vacuo*, diluted, and adjusted to a final concentration of 50 mM sodium phosphate buffer with a pH of 4.2. In the third purification step, the eluate was separated on a SP Sepharose cation exchange column (GE Healthcare, Germany) with a linear 1 M NaCl gradient in 50 mM sodium phosphate buffer on a Äkta FPLC (GE Healthcare, Germany). The two active compounds were eluted separately at 120 mM and 280 mM NaCl concentration respectively. The final purification and desalting of each peak was carried out by reversed phase preparative HPLC (RP-HPLC) (Bischoff, Leonberg, Germany) on a nucleosil 100 C-18, 8×250 mm column (Machery Nagel, Düren, Germany) with a linear water acetonitrile (containing 0.1% TFA) gradient of 0% to 60% in 25 min. Purified compounds were lyophilized and stored at −20°C. Qualitative analysis was carried out on an Agilent 1200 HPLC system (Agilent technologies, Waldbronn, Germany) and a RP-HPLC Waters xBridge C18, 5 mm, 4.6×150 mm column. Compounds were eluted with a 15 min linear gradient of 0.1% phosphoric acid to acetonitrile at a flow rate of 1.5 ml/min. For structure elucidation, mass spectrometry was carried out on GC-MS and FT-ICR MS (Bruker, ApexII). NMR spectra were measured in d_4-methanol and recorded on a Bruker AMX600 spectrometer (600 MHz for ^1H, 150 MHz for ^{13}C), solvent was used as internal standard ($\delta_{H/C}$ 3.31/49.15 for MeOH-d_4).

Assessing QS-regulated compounds in *S. marcescens*, *P. aeruginosa*, *C. subtsugae*, and bioluminescence in *V. harveyi* in co-cultivation with *Staphylococcus sp.*

Overnight cultures of *S. aureus* and *S. delphini* cells were diluted in LB medium containing 0.3% glucose to an OD_{578} value of 1.0 (we also tried 0.1, 0.5, but OD 1.0 results were most pronounced), incubated for 4 h at 37°C, then co-cultivated at 30°C with *S. marcescens* or *P. aeruginosa*; each strain was inoculated with a starting OD_{600} of 0.01. After 24 h of cultivation, prodigiosin of *S. marcescens* was extracted from the cell pellets by ethanol acidified with 4% of 1 M hydrochloric acid and then quantified by $A_{534\ nm}$ determination. Pyocyanin in the supernatant was determined by its absorption maximum at 520 nm [58]. For bioluminescence tests, overnight-cultured *V. harveyi* was diluted to an OD_{578} of 1.0 and co-cultivated with an equal amount of *S. aureus* or *S. delphini* cells in LB-MB (LB medium containing 0.1% glucose mixed with equal volumes of MB) for 18 h. Overnight cultures of *S. aureus* and *S. delphini* cells were diluted to an OD_{578} of 0.1, incubated for 4 h at 37°C, then co-cultivated at 30°C with *C. subtsugae*, which was diluted to a final OD_{600} value of 2; this high OD was necessary to pronounce violacein production, whose expression is dependent on AHL at higher cell density. After 24 h of incubation, cell pellets were collected and resuspended in water. Cells were lysed by 10% sodium dodecyl sulfate and incubated for 5 min at room temperature. Violacein was quantitatively extracted from the cell by adding water-saturated butanol. The butanol phase containing the violacein was collected and determined at its absorption maximum at 585 nm.

Production of yayurea A and B in *S. delphini* and their effect on bacterial growth

S. delphini was inoculated to a starting OD_{578} of 0.1 and incubated at 37°C. During the 24 h incubation, cell density was followed by OD_{578} and the active compounds in the supernatants were determined by RP-HPLC and phosphoric acid – acetonitrile gradient as described above. The Gram-negative representatives, *E. coli*, *P. aeruginosa*, *S. marcescens*, *V. cholerae* and *V. harveyi* were inoculated to OD_{600} of 0.1 and grown in LB for 2 h. After that time, 1 mg/ml yayurea A or B (as solution in H_2O) or an equal volume of water as negative control was added. Cells were either incubated at 37°C (*S. aureus* or *S. delphini*) or 30°C (*E. coli*, *S. marcescens*, *V. harveyi*, *V. cholerae* and *P. aeruginosa*). Cell density was followed for 24 h.

Activity assays for QS-regulated compounds, bioluminescence, and biofilm formation in Gram-negative bacteria

Pyocyanin production. An overnight culture of *P. aeruginosa* PAO1 was diluted with LB medium to an OD_{600} of 0.1. The (Z-)-4-*bromo*-5-(bromomethylene)-2(5H)-furanone (Sigma), yayureas and tetracycline were dissolved in 10% DMSO. 190 µl diluted PAO1 was cultured in 96-well plates with 10 µl different concentrations of pure yayurea A, B, furanone, or 10% DMSO (negative control) at 30°C. After 24 h of incubation, cell density was measured at 600 nm and pyocyanin was isolated as described previously [58]. To measure pyocyanin production, the supernatants were subjected to HPLC separation on a Nucleosil 100, C-18 column and a 0–100% Water-ACN Gradient (water containing 0.1% phosphoric acid) in 15 min at a flow rate of 1.5 ml/min. The content of pyocyanin was determined at its absorption maximum at 520 nm [58]. The relative pyocyanin production was calculated as the ratio of the amount of pyocyanin content from the HPLC measurements to cell density.

Prodigiosin production. *S. marcescens* cells were inoculated from an overnight culture to a starting OD_{600} of 0.1. Then 100 µl of the diluted culture was mixed with 100 µl of different amounts of the purified compounds or water in wells of 96-well microtiter plates and incubated for 24 h at 28°C. Cell density was measured at 600 nm. Prodigiosin was extracted as described above and the relative prodigiosin production was calculated as the ratio between the amount of prodigiosin at its absorption maximum at 534 nm and cell

density values at 600 nm. The prodigiosin suppressing effect of yayurea A and B was determined (H_2O was used as control).

Biofilm formation assay. Biofilm formation assays were performed as described previously [34]. A full-grown culture of *P. aeruginosa* PAO1 or *S. aureus* was diluted with LB medium containing 0.3% (w/v) glucose to an OD_{600} of 0.1. 100 µl of the dilution was mixed with 100 µl of the yayurea solutions with the indicated concentrations, and incubated in 96-wellplates at 37°C for 24 h. To determine the biofilm formation, planktonic cells were discarded and the plates gently washed with PBS and air-dried for 30 min. The wells were stained with 200 µl of a 0.1% crystal violet solution at room temperature for 30 min. The stained biofilm was rinsed with distilled water followed by the addition of 200 µl DMSO. Absorbance of crystal violet as indicator for biofilm-forming bacteria was measured at 590 nm. The effects of the compounds were evaluated for which the sample with water was set to 100%.

Bioluminescence. An overnight culture of *V. harveyi* was diluted to an OD_{600} of 0.1 and grown with yayurea A, B, or water for 24 h at 28°C. Bioluminescence was determined in a Tecan infinite M200 plate reader (Tecan, Groedig, Austria). Relative luminescence was normalized by the OD_{600} values. The effects of the compounds were calculated by the relative luminescence, for which the sample with water was set to 100%.

Investigation of the QS-target(s) of yayureas *in vitro*

Production of QS-receptors and QS-molecules. Inverted membrane vesicles were prepared using *E. coli* strainTKR2000 expressing plasmids pNKN, pNKQ, and pNKS encoding wild type LuxN, LuxQ, and CqsS (each with a C-terminal His-tag), respectively. Inverted membrane vesicles were prepared as described by [36]. LuxP was produced in, and purified from, *E. coli* MDAI-2 transformed with the plasmid pGEX_LuxP as described before [59]. LuxU was produced and purified as described before, using *E. coli* JM109 transformed with plasmid pQE30LuxU-6His [36]. Synthetic auto-inducer, HAI-1, (from the University of Nottingham) was dissolved in a minimal volume of acetonitrile [10% (v/v)], diluted with water to a concentration of 100 mM and stored at −20°C.

Phosphorylation assays. Each QS-receptor kinase was tested as full-length membrane integrated proteins in inverted membrane vesicles. The buffer used for phosphorylation reactions was as follows: 50 mM Tris/HCl pH 8.0, 10% (v/v) glycerol, 500 mM KCl, 2 mM DTT. Each phosphorylation reaction contained equimoloar receptor concentrations [the total protein concentration were accordingly adapted: 3.2 mg/ml (LuxN), 5.5 mg/ml (LuxQ), and 5.2 mg/ml (CqsS) membrane proteins], and purified LuxU at 0.1 mg/ml. For phosphorylation of LuxQ, LuxP was added at a concentration of 0.4 mg/ml. LuxP integration into LuxQ-bearing membrane vesicles was triggered by several cycles of thawing and freezing. When indicated, yayurea A or B were added to the reaction mix at a final concentration of 220 µg/ml, and HAI-1 at 20 µM. The reaction was started by adding radio labeled Mg^{2+}-ATP, typically 100 µM [γ-^{32}P]ATP (specific radioactivity of 0.94 Ci/mmol; Perkin Elmer) and 110 µM $MgCl_2$. The reaction was stopped after 1 and 10 minutes by adding SDS Laemmli-loading buffer followed by separation of the proteins on SDS-polyacrylamide gels. Gels were dried at 80°C on filter paper, exposed to a phosphoscreen for at least 24 h, and subsequently scanned using a PhosphorImager SI (GE Healthcare). The gels presented are representative of at least three independent sets of experiments.

Supporting Information

Figure S1 Mass spectra of Yayurea A and B. Mass spectrometry was carried out on GC-MS and FT-ICR MS (Bruker, ApexII).
(TIF)

Figure S2 Influence of yayurea A and B on growth. Growth curve of *S. marcescens*, *P. aeruginosa*, *V. harveyi* and *V. cholerae* in BM with 1000 µg/ml yayurea A or B or equal volume of water. Arrow indicates time point (after 2 h) of the addition of compounds to the growing culture. All the measurements were made in triplicate. Bars indicate standard deviation, SD.
(TIF)

Figure S3 Phylogenetic tree among *Staphylococcus* species. The tree is based on 16S rRNA relationships according to [45]. The phylogenetic position of the 'intermedius group' composed of *S. intermedius*, *S. pseudintermedius*, and *S. delphini* is marked in bold. Based on a combinatin of 16S rRNA and multilocus data, the group was recently complemented by the next related species *S. lutrae*, *S. schleiferi* subsp. *schleiferi* and *S. schleiferi* subsp. *coagulans* [37]. All members of this group produce yayurea A and B.
(TIF)

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Author Contributions

Conceived and designed the experiments: FG SG KJ. Performed the experiments: YYC MN MW LP. Analyzed the data: YYC MN MW LP FG SG KJ. Contributed reagents/materials/analysis tools: FG SG KJ. Wrote the paper: FG YYC MN MW LP SG KJ.

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Category by field: Microbiology> Microbial biochemistry> Metabolite analysis
Category by field: Biochemistry> Other compound analysis> Metabolite analysis
Category by organism: Bacteria> Staphylococcus > *Staphylococcus delphini* > Other compound analysis

Purification and Structural Analysis of QS-inhibiting Compounds from *Staphylococcus delphini*

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[Abstract] The knowledge that many pathogens rely on cell-to-cell communication mechanisms known as quorum sensing, opens a new disease control strategy: quorum quenching. Here we present a purification protocol for molecules excreted by a group of Gram-positive zoonotic pathogen bacteria, the '*Staphylococcus intermedius* group', that suppress the quorum sensing signaling and inhibit the growth of a broad spectrum of Gram-negative beta- and gamma-proteobacteria. These compounds were isolated from *Staphylococcus delphini*. They represent a new class of quorum quenchers with the chemical formula *N*-[2-(*1H*-indol-3-yl)ethyl]-urea and *N*-(2-phenethyl)-urea, which we named yayurea A and B, respectively. These substances can be isolated and purified from the culture supernatant using this upscalable purification method.

Materials and Reagents

1. *Staphylococcus delphini* DSMZ20771 strain (DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSM number: 20071)
2. Tryptic Soy Broth (TSB) (Sigma-Aldrich, catalog number: T8907)
3. Amberilite XAD-16 resin (Sigma-Aldrich, catalog number: 1-0379)
4. Methanol
5. Acetic acid (Merck KGaA)
6. Amberilite IRC 50 cation exchange resin (SERVA Electrophoresis, catalog number: 40501)
7. Sodium hydroxide
8. Ethanol
9. 50 mM and 1M Sodium phosphate buffer
10. SP Sepharose cation exchange column (GE Healthcare, catalog number:17-5161-01)
11. Sodium chloride
12. Trifluoroacetic acid (TFA) (Sigma-Aldrich, catalog number: T6508)
13. Phosphoric acid for HPLC (Sigma-Aldrich, catalog number: 79606)
14. Acetonitrile for HPLC (Mallinckrodt Baker, catalog number: 9012)

Equipment

1. 37 °C shaking incubator (INFORS)

2. Centrifuge (Eppendorf)
3. Rotary evaporator (BÜCHI Labortechnik AG)
4. Äkta FPLC equipped with P-900, UV-900, PH/C-900 (GE Healthcare)
5. Preparative HPLC System equipped with Bischoff HPLC compact pump QC-P 2250 and Multiwavelength detector QC-1157 (Bischoff)
6. Nucleosil 100 C-18 (8 × 250 mm column) (MACHEREY-NAGEL, catalog number:715332.80)
7. Agilent 1200 series HPLC system (Agilent)
8. Waters XBridge C18 (5 mm, 4.6 × 150 mm column) (Waters, part number:186003116)

Procedure

1. *S. delphini* is cultivated in 100 ml TSB at 37°C on a shaking incubator at 150 rpm for 20 h.
2. Cells are centrifuged at 5000 rpm (4500 x g) at 4ºC for 10 min and the supernatant is applied on to a column filled with 10 ml Amberilite XAD-16 resin.
3. The column is first washed with 5 bed volumes each of milliQ water, then with 40% and 60% methanol and finally eluted with 80% methanol containing 5% acetic acid at a flow rate of 10 bed volumes per hour.
4. The eluate is evaporated using a rotary evaporator until all methanol is removed.
5. The eluate is resuspended with 50ml water and the pH adjusted to 7.0 with 1 M NaOH. It is then applied on to a column filled with 10ml Amberilite IRC-50 cation exchange resin.
6. The column is washed first with water, then with 70% Ethanol and eluted with 80% Ethanol acidified with 5% acetic acid each with 5 bed volumes at a flow rate of 10 bed volumes per hour.
7. The eluate is concentrated using a rotary evaporator until all methanol is removed.
8. The eluate is diluted with water and adjusted to a final concentration of 50 mM sodium phosphate using 1 M sodium phosphate buffer and pH is lowered to 4.2 using 85% phosphoric acid.
9. In the third purification step, the eluate is separated on a 5 ml SP Sepharose cation exchange column with a linear 0 to 1 M NaCl gradient in 50 mM sodium phosphate buffer on an Äkta purifier FPLC at a flow rate of 3 ml/min.
10. The final purification and desalting of each peak is carried out by reversed phase preparative HPLC (RP-PHPLC) on a nucleosil 100 C-18, 8 × 250 mm column with a linear water acetonitrile (containing 0.1% TFA) gradient of 0% to 60% in 25 min.
11. Purified compounds are lyophilized and stored at −20 °C.
12. Qualitative analysis is carried out on an Agilent 1200 HPLC system and a RP-HPLC Waters xBridge C18, 5 mm, 4.6 × 150 mm column. Compounds are eluted with a 15 min linear gradient of aquaeus phosphoric acid (0.1% vol/vol) to acetonitrile at a flow rate of 1.5 ml/min and detected at 210 nm.

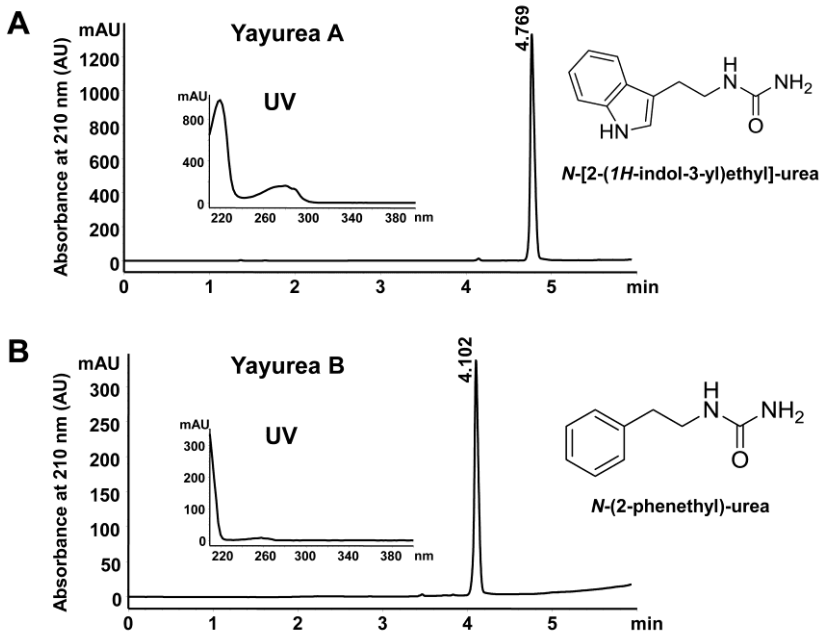

Figure 1. RP-HPLC profile, UV-spectrum and structures of the two QS-inhibitors purified from *S. delphini*. A. QS-inhibitor, *N*-[2-(1H-indol-3-yl)ethyl]-urea (yayurea A). B. QS-inhibitor, *N*-(2-phenethyl)-urea (yayurea B). RP-HPLC is carried out on an Agilent 1200 and Waters xBridge C18, 5 mm, 4.6 x 150 mm column; compounds are eluted with a 15 min linear gradient of 0.1% phosphoric acid to acetonitrile at a flow rate of 1.5 ml/min.

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The role of serum proteins in *Staphylococcus aureus* adhesion to ethylene glycol coated surfaces

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Abstract

Bacterial adhesion on implants is a first step in the development of chronic foreign body associated infections. Finding strategies to minimize bacterial adhesion may contribute to minimize such infections. It is known that surfaces with oligo-ethylene-glycol (EG_3OMe) or poly-ethylene-glycol (PEG2k) terminations decrease unspecific protein adsorption and bacterial adhesion. However, little is known about the influence of serum and its components on bacterial adhesion. We therefore prepared two coatings on gold surface with HS-$(CH_2)_{11}EG_3OMe$ (EG_3OMe) and PEG2k-thiol and studied the role of bovine serum albumin (BSA), γ-globulins, and serum on *Staphylococcus aureus* adhesion. While BSA and lysozyme showed no adherence even when applied at very high concentrations (100 mg/ml), γ-globulins adsorbed already from 10 mg/ml on. The adsorption of γ-globulins was, however, significantly decreased when it was mixed with BSA in a ratio of 3:1, as it is in the serum. Pretreatment of EG_3OMe and PEG2k coatings with γ-globulins or serum strongly promoted adherence of *S. aureus* when resuspended in buffer, suggesting that γ-globulins play a pivotal role in promoting *S. aureus* adhesion by its IgG binding proteins; the finding that a *spa*-deletion mutant, lacking the IgG binding protein A, showed decreased adherence corroborated this. Similarly, when *S. aureus* was pretreated with serum or γ-globulins its adherence was also significantly decreased. Our findings show that particularly γ-globulins bind to the coated surfaces thus mediating adherence of *S. aureus* via its protein A. As pretreatment of *S. aureus* with serum or γ-globulins significantly decreased adherence, treatment of patients with γ-globulins before implant surgery might lower the risk of implant-associated infections.

Keywords: Albumin, bovine serum, γ-globulins, oligo (ethylene glycol) (EG_3OMe) thiol surface coating, poly(ethylene glycol) (PEG2k) thiol surface coating, *Staphylococcus aureus* adhesion

Introduction

Infections related to indwelling medical devices are one of the major causes of persistent clinical infections (Costerton et al., 1999). These infections are frequently complicated by biofilm formation, during which the pathogenic microorganisms adhere to the surfaces of the medical devices and develop a thick, multilayered slimy matrix (Cramton and Götz, 2004; Cramton et al., 1999; Gross et al., 2001; Kropec et al., 2005; Rohde et al., 2005). Once the biofilm is formed, the embedded microorganisms are highly resistant to multiple antibiotics

and host immune defense (Gray et al., 1984; Stewart and Costerton, 2001). Often, the only way to eradicate such infections is to surgically remove the infected indwelling device. The nonspecific and reversible adhesion of microorganisms to the surfaces of the biomaterials is the critical step in biofilm infections. Pathogenic microorganisms have employed a variety of factors for successful adhesion. As the most common biofilm associated microorganisms, *Staphylococcus epidermidis* and *Staphylococcus aureus* exploit major autolysin (Heilmann et al., 1997), surface proteins SasG, SasC (Roche et al., 2003; Schroeder et al., 2009) as well as teichoic acids (Gross et al., 2001) to initiate adhesion. Immunoglobulin-, fibrinogen-, fibronectin-, or collagen- binding proteins promote the adhesion of *S. aureus* to a surface if the surface is coated with the corresponding matrix proteins (Götz and Peters, 2000).

Preventing the initial adhesion of microorganisms to the surfaces of biomaterials is thought to be an efficient way to treat implant-associated infections. Apart from attempts to target bacterial adhesion factors by selected antibiotics (Saising et al., 2012), much attention has been paid in recent years to engineering biomaterial surfaces to become more resistant to bacterial adhesion. Self-assembled monolayer (SAMs) based surface coatings with oligo-ethylene-glycol (OEG) or poly-ethylene-glycol (PEG) terminations render surfaces resistant against non-specific protein adsorption and bacterial adhesion, which appears to be a promising approach (Pale-Grosdemange et al., 1991; Prime and Whitesides, 1993). The mechanisms for the protein resistance property of PEG coated surfaces are proposed to be free energies of steric repulsion, hydrophobic interactions and van-der-Waals interactions (Jeon and Andrade, 1991). Both grafting density and chain length are critical parameters for the efficiency of PEG in preventing protein adsorption and bacterial adhesion (Holmberg et al., 1993). Due to the short chains and highly ordered conformation, the mechanisms of the protein resistant property of OEG coatings are different in many parameters such as the surface coverage, the conformation and the types of end groups (Love et al., 2005; Schreiber, 2004; Ulman, 1996).

As the implant-associated infections often occur in complex biological systems, it is important to assess the influence of environmental factors on the interaction between bacteria and the biomaterial's interfaces. Blood plasma is one of the most relevant environmental factors for medical device related infections. Under clinical conditions, significant protein adsorption from blood plasma has been observed for various modified interfaces (Benesch et al., 2001; Olsson et al., 1992). While previous studies focus either on the resistance against bacterial adhesion in single protein solution (Cheng et al., 2007) or on plasma protein adsorption without testing bacterial adhesion (Zhang et al., 2008), an advanced understanding

of how plasma proteins affect the bacterial adhesion to the interfaces is largely missing (Deng et al., 1996; Katsikogianni and Missirlis, 2004).

In the current work, we prepared EG_3OMe ($HS\text{-}(CH_2)_{11}EG_3OMe$, one kind of OEG coating) and PEG2k (one kind of PEG coating) coated surfaces that had good physicochemical properties in preventing protein adsorption and *S. aureus* adhesion. We then compared the protein adsorption behavior of bovine serum albumin (BSA), γ-globulins and whole bovine serum at their physiological concentrations. Further, the effects of BSA, γ-globulins and serum on initial adhesion of *S. aureus* to the EG_3OMe and PEG2k coated surfaces were investigated. Our results showed that albumin, γ-globulins and serum had distinct protein adsorption behaviors and consequently different effects on bacterial adhesion. While γ-globulins readily adhered to the coated surfaces, albumin did not adhere and even suppressed γ-globulin adherence in mixed solution. Protein A played a crucial role in adherence when the coatings were pretreated with serum or γ-globulins. However, when *S. aureus* cells were pretreated with serum or γ-globulins to saturate protein A adherence was significantly decreased.

Materials and Methods

Preparation of OEG and PEG coatings on gold (Au) surfaces

Silicon wafers (Si<111>) coated with a 5 nm Ti layer and a 200 nm evaporated Au layer were used as substrates for coating. The wafers were cleaned by rinsing in Milli-Q water (18.2 MΩ cm, Millipore) and ethanol (99.9%, Riedel de Haen), dried in an argon stream, treated with ozone producing UV-light for 20 min and rinsed with Milli-Q water again. The coating procedure was performed directly after the cleaning. For the OEG coating preparation, a 500 μM solution of $HS\text{-}(CH_2)_{11}EG_3OMe$ (EG_3OMe) or EG6OMe in ethanol was used with an immersion time of 24 h (Skoda et al., 2007; Zorn et al., 2011). For the PEG coatings, a 50 μM solution of PEG2k or PEG5K in N, N-Dimethylformamide (DMF) was used with an immersion time of 48 h (Schilp et al., 2009). After removal of the surfaces from the thiol stock solutions, the coated surfaces were rinsed with pure ethanol and dried with a nitrogen stream (Skoda et al., 2007; Zorn et al., 2010). The coated samples were stored in the dark in a nitrogen environment. The structures of EG_3OMe and PEG2k are shown in Fig. 2.

Protein adsorption experiment

The EG_3OMe and PEG2k coated surfaces were rinsed briefly in Milli-Q water and then dried in an Argon stream. Directly after this procedure the surfaces were incubated in a protein solution for 15 min, washed briefly with Milli-Q water and dried with an Argon stream. Proteins tested were lysozyme, fibrinogen, bovine serum albumin (BSA) and bovine γ-globulins (Sigma-Aldrich, Germany). In the first experiment, BSA, lysozyme and fibrinogen were tested at a concentration of 1 mg/ml. In the second experiment, individual proteins of different concentrations (1, 10, 20, 50, 100 mg/ml) were tested. Lastly, protein adsorption of a mixture of BSA (end concentration 30 mg/ml) and γ-globulins (end concentration 10 mg/ml) in a ratio of 3:1 and the whole bovine serum were studied.

Protein desorption and analysis by SDS-PAGE

Gold wafer samples coated with each of the proteins BSA, γ-globulins and mixture of both were washed with 1M Tris/HCl pH 8.4 buffer containing 0.1% SDS. After washing the surface with the buffer repeatedly, the wafers were further shaken in the same buffer in a sonication bath to enhance desorption of the coated proteins. The solution was then mixed with laemmli buffer and analysed by SDS-PAGE.

Bacterial adhesion assay

S. aureus SA113 (pC-tuf-ppmch) constitutively expresses red fluorescent protein mCherry (Mauthe et al., 2012) was used in this work. In addition *S. aureus* SA113 WT (pC-tuf-gfp) and the protein A deficient mutant *S. aureus* SA113 Δ spa (pCX-pp-sfgfp), both expressing the green fluorescent protein (gfp) were used as well. Bacteria were cultivated overnight at 37 °C and 120 rpm in basic medium (BM) composed of 1% peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose and 0.1% K_2HPO_4. The overnight culture was inoculated into fresh medium with an adjusted initial OD_{578} of 0.1 and grown to a density of 1.0. Bacterial cells were then centrifuged and washed three times with sterile phosphate buffered saline (PBS). Subsequently the bacteria were resuspended in PBS or bovine serum to OD_{578} of 1.0. Sterile 12-well cell culture plates (Greiner bio-one), onto which EG_3OMe and PEG2k coated samples were placed, were each filled with 4 ml bacteria suspensions. The plates were incubated at 37°C on a rotary shaker at 100 rpm. After 1 h incubation, the samples were washed three times for five minutes in fresh PBS buffer and degassed Milli-Q water and finally dried with an Argon stream. In the pre-incubation assay, the EG_3OMe and PEG2k coated samples were each pre-incubated for 1 h with PBS, BSA (20 mg/ml), γ-globulins (60 mg/ml) and bovine serum (100%), in turn, before incubating with the bacteria suspension.

Atomic Force Microscopy (AFM)

Experiments were performed with a NanoWizard 3 AFM (JPK) in the tapping mode with a line scan rate of 1 Hz and a resolution of 512×512 pixels. Scans were performed with a size of 1×1 μm^2, 2×2 μm^2, 5×5 μm^2 and 10×10 μm^2 to determine the average surface roughness. The AFM images were treated with "Gwyddion" and the surface roughness and height distribution were obtained. The mean-squared roughness (MSR) is given by the standard deviation of the z-values for the surface height. It is quantified by the vertical deviations of a real surface from its ideal form; if these deviations are large, the surface is rough, and if they are small the surface is smooth.

Polarization Modulation Infrared Reflection Absorption Spectroscopy (PMIRRAS)

PMIRRAS measurements were performed on a Vertex70 Spectrometer (Bruker, Ettlingen, Germany) equipped with a PMA50 extension (Bruker) featuring a photoelastic modulator, purged with dry air. The spectra were recorded with a resolution of 4 cm^{-1} and 1024 scans per measurement. The spectra were exported from the Bruker data acquisition program OPUS and baseline corrected in Igor Pro (WaveMetrics, USA). The area, amplitude, width and position of the modes in the fingerprint region were determined by fitting with a Gaussian function (Roosen-Runge et al., 2010). Details of setup and data analysis have been described elsewhere (Skoda et al., 2009). The detector was fixed at an angle of 75.8°. The sample holder position was rotated to an angle of 82.9° to have the maximum interferogram signal (Skoda et al., 2007). These settings were used for all measurements. Surface-bound proteins were identified by the characteristic amide I (CO and N-H groups) absorption (the stretching vibrations of the peptide carbonyl group), which lies in the range of 1600-1700 cm^{-1}.

Fluorescence Microscopy

Experiments were performed using a Leica DM5500 B microscope. Images were captured with the Leica DFC360 FX high sensitivity monochrome digital camera. The fluorescence coverage from each photo was calculated with the software "ImageJ", using a size of 1 μm^2 as an input parameter.

Results

Morphology and structure of EG_3OMe and PEG2k coated surfaces

Both uncoated as well as EG_3OMe and PEG2k coated gold (Au) surfaces were analyzed by AFM with respect to morphology and mean-squared roughness (MSR) (Fig. 1). The sputtered Au surfaces on silicon wafers had a grain-like morphology with a domain size in the range of 50 -70 nm in diameter, MSR of 2.19±0.01 nm (Fig. 1A). These Au surfaces on silicon wafers were coated with EG_3OMe and PEG2k. The EG_3OMe coated surfaces showed a similar grain-like morphology with a similar domain size of ~60 nm in diameter (Fig. 1B). However, the surface became more flattened and more smooth as indicated by a decrease of the MSR from 2.19±0.01 nm (Fig. 1A) to 1.58±0.02 nm. When the gold surface was coated with PEG2k the surface became even smother as indicated by a drop of the MSR to 0.82±0.01 nm (Fig. 1C). An enlargement of the PEG2k coated surface is shown in Fig. 1D, which indicates the formation of larger domains on PEG2k coated surface.

EG_3OMe and PEG2k coated surfaces did not adsorb lysozyme, fibrinogen and BSA at low concentration

The internal structures of EG_3OMe and PEG2k coatings were characterized by PMIRRAS (Fig. 2). The spectrum of EG_3OMe coatings in air showed a single peak at ~1130cm^{-1} (C-O-C stretching mode) (Fig. 2A, black spectrum, red arrow), indicating that the dominant conformation was the ordered helical conformation. The spectrum of PEG2k coatings had a dominant peak at ~1130cm^{-1} (Fig. 2B, black spectrum, red arrow) with a shoulder at ~1144cm^{-1} (Fig. 2B, black spectrum, blue arrow), suggesting a mixture of the ordered helical and the less ordered all-trans conformations, but dominated by helical conformation in the long polymer chain (Matsuura and Miyazawa, 1969; Miyazawa et al., 1962).

To test the protein adsorption properties of EG_3OMe and PEG2k coated surfaces they were incubated with selected blood proteins at a concentration of 1 mg/ml. The proteins vary in molecular weight (M_w) and isoelectric point (pI). Lysozyme is a small protein (M_w=14.3 kD, pI=12) that is positively charged under physiological pH (Holmlin et al., 2001). Fibrinogen is a large protein (M_w=340 kD) with negative charge (pI=6.0). BSA has an intermediate protein size (M_w=68kD) and a pI of 4.6. PMIRRAS spectra of freshly prepared EG_3OMe and PEG2k coatings before and after incubating with protein solutions were shown in Fig. 2. In all cases, no significant amide I band (1600-1700 cm^{-1}) was visible after incubating with proteins (Fig. 2, boxed square). The slight deviation from the baseline might be due to the background subtraction. All other features of the absorption bands from the thiols remained identical, indicating no conformation change of thiol molecules in the

coatings after exposure to proteins. The amide I band is associated with the stretching vibrations of the peptide carbonyl group, which has been widely used as an indicator to monitor protein adsorption at interfaces (Barth and Zscherp, 2002). Our results showed that both EG_3OMe and PEG2k coatings are largely inert to protein binding, which is consistent with a previous report (Zhu et al., 2001) .

At high concentration (physiological) only γ-globulins and serum showed strong adsorption to the coated surfaces but not albumin

As shown above (Fig. 2), lysozyme, fibrinogen and BSA hardly adsorbed to EG_3OMe and PEG2k coated surfaces when applied at a concentration of 1 mg/ml. However, in blood serum the concentration is much higher. In blood serum, the overall protein concentration is about 70 mg/ml. The two most abundant proteins are albumin ~60% (35 – 50 mg/ml) and γ-globulins ~18% (10 – 15 mg/ml) (Burtis and Ashwood, 1999). We thus extended our experiments by increasing the protein concentrations (Fig. 3A) and also using a combination of BSA and γ-globulin.

PMIRRAS spectra indicated that the adsorption of BSA and lysozyme was very low for both coatings, even at 100 mg/ml (Fig. 3B, purple and green lines). In contrast, the adsorption of γ-globulins quickly reached a maximum at concentrations of 20 to 50 mg/ml, the adsorption on PEG2k coatings was lower but continuously increased with protein concentration (data not show).

When combinations of BSA and γ-globulins with a ratio of 3:1 were tested, protein adsorption was significantly suppressed at all concentrations, indicating that BSA suppressed the adsorption of γ-globulins (Fig. 3B, red line). The suppressive effect of BSA on γ-globulin coating is also illustrated in SDS PAGE (Fig. 3C) and Fig. 3D.

However, when whole bovine serum was tested the EG_3OMe and PEG2k coating showed different results (Fig. 3D). With EG_3OMe coatings, the total protein adsorption of serum was low and similar to that of BSA and γ-globulins mixture. With PEG2k coatings, the protein adsorption of serum was significantly increased compared to BSA and γ-globulins mixture (Fig. 3D, yellow bars), indicating that PEG2k coatings adsorbed some other proteins in the serum apart from BSA and γ-globulins.

Our results show that at physiological blood protein concentrations, the adsorption behavior of BSA, γ-globulins and whole bovine serum varies with the coating. BSA showed little adsorption on both coatings, whereas γ-globulins showed strong adsorption. BSA could suppress γ-globulins adsorption when it was mixed with γ-globulins in a ratio of 3:1, which

represents serum conditions. Whole serum had strong adsorption on PEG2k coatings, but not on EG_3OMe coatings, suggesting that some other serum proteins were adsorbed on the PEG2k coatings.

Bacteria resuspended in buffer did not adhere EG_3OMe and PEG2k coated surfaces

The EG_3OMe and PEG2k coated surfaces were also tested for bacterial adhesion. *S. aureus* (pC-tuf-ppmch) that constitutively expressed red fluorescent protein mCherry was suspended in PBS to an OD_{578} of 1.0 and incubated with coated and uncoated surfaces for 1 h. The uncoated gold surface was heavily covered with cells, while the EG_3OMe and PEG2k coated surfaces were hardly covered with bacteria (Fig. 4A1, 2 and 3). This result was confirmed by the quantitative analysis of the fluorescence coverage of the visual field (Fig. 6, first block, 'PBS+PBS'). Our results show that EG_3OMe and PEG2k coated surfaces were largely inert to bacterial adhesion. We also tested other coating material with greater length, EG6OMe and PEG5K, and they showed similar results as EG_3OMe and PEG2k (data not shown).

Pre-incubation of EG_3OMe and PEG2k coated surfaces with γ-globulins or serum promoted S. aureus adhesion

As implant material usually comes in contact with plasma components it is important to know what influence they have on bacterial adhesion. To address this question, we used two experimental settings. In the first experiment, the coated surfaces were pre-incubated with buffer (PBS control), BSA (20 mg/ml), γ-globulins (50 mg/ml) and bovine serum for 1 h. Subsequently the surfaces were incubated with *S. aureus* (pC-tuf-ppmch) suspended in PBS (OD_{578} of 1.0) for another hour. After removal of unbound bacteria by PBS-washings, bacterial adhesion was visualized with fluorescence microscopy. With this experiment, we aimed to investigate whether there is a correlation between protein adsorption and bacterial adhesion. When the coated surfaces were pre-incubated with BSA, which showed almost no adsorption itself but significantly prevents adsorption of γ-globulins in mixed BSA-γ-globulins samples (Fig. 3B), there was no adherence of *S. aureus* observed compared to the PBS control (Fig. 4A4). The results were comparable to untreated coatings as shown in Fig. 4A2 and 3. However, if the coated surfaces were pre-incubated with γ-globulins, which readily adsorbs to the coated surfaces (Fig. 3), the adhesion of *S. aureus* was significantly promoted (Fig. 4A5). The adherence of *S. aureus* to EG_3OMe coating was higher than to PEG2k coating, which correlates with the higher adsorption of γ-globulins to EG_3OMe

coating (Fig. 3). In the quantitative fluorescence assay the amount of bacteria attached to EG_3OMe coatings was five-fold higher than that on PEG2k coatings (Fig. 6: γ-Globulin+PBS). If the coated surfaces were pre-incubated with bovine serum, *S. aureus* adhesion was also enhanced on both coatings (Fig. 4A6).

As shown in Fig. 6 (block 'serum+PBS'), twice as much bacteria were adhered to the EG_3OMe coatings than to PEG2k coatings, which correlated with the protein adsorption assay (Fig. 3). There was a clear correlation between the amount of γ-globulins bound to the surfaces and the amount of bacterial adhesion. As shown in Fig. 6 (blocks 'γ-Globulins+PBS' and 'serum+PBS'), the bacteria attached after pre-incubating with 60 mg/ml γ-globulins were six-fold higher than that after pre-incubating with serum where the γ-globulins were about 10 mg/ml. We assume that the adherence of *S. aureus* to serum-coated surfaces was essentially due to the relatively high content of γ-globulins in the serum (approximately 20% of total proteins are γ-globulins). We also tested human serum, which had the same effect as bovine serum (data not show).

Pre-incubation of S. aureus with serum remarkably inhibited bacterial adhesion at EG_3OMe and PEG2k coated surfaces pretreated with γ-globulins or bovine serum

In the above (Fig. 4A) experiment we resuspended *S. aureus* (pC-tuf-ppmch) in PBS, which represents an artificial situation, because under in vivo conditions *S. aureus* comes in close contact with serum. In order to better mimic the in vivo situation we resuspended *S. aureus* in bovine and human serum and carried out the same adherence assay with EG_3OMe and PEG2k coatings pre-treated with BSA, γ-globulins or bovine serum as described above (Fig. 4B).

Interestingly, resuspending *S. aureus* with bovine serum remarkably reduced *S. aureus* adhesion to EG_3OMe and PEG2k coatings pre-treated with γ-globulins or bovine serum (Fig. 4B5 and 6). Apparently serum masks the *S. aureus* cell surface with serum components, most likely γ-globulins, thus reducing its binding capacity to coatings pre-treated with γ-globulins or serum. No binding of serum resuspended *S. aureus* was observed with coatings pre-treated with BSA (Fig. 4B4), or with un-pretreated EG_3OMe and PEG2k coatings (Fig. 4B2 and 3). Even with the uncoated Au surface *S. aureus* (pC-tuf-ppmch) adherence was significantly decreased (Fig. 4B1). The same effect was observed when *S. aureus* (pC-tuf-ppmch) was resuspended with human serum (not shown). The results are summarized in the quantitative analysis diagram (Fig. 6).

The pretreatment of EG_3OMe and PEG2k coatings with γ-globulins or bovine serum caused massive adhesion of *S. aureus*. We assume that might be due to the fact that *S. aureus* harbors high immunoglobulin-binding activity by its cell-wall bound protein A (Spa) (Löfdahl et al., 1983; Uhlen et al., 1984). The 'second immunoglobulin-binding protein' (Sbi) is secreted (Zhang et al., 1998) and should not play a major role in adherence. To prove the hypothesis, *S. aureus* SA113Δspa (pCX-pp-sfgfp), which is a Protein A deficient mutant strain, was used for the tests on uncoated Au or PEG5K coated surfaces. When the bacteria were resuspended in PBS, the uncoated Au surface was heavily covered by both strains (Fig. 5A1 and B1). If WT and Δspa mutant were resuspended in buffer there was no adherence to PEG5K coated surface (Fig. 5A2 and B2). Was the PEG5K coated surface pretreated with γ-globulins the WT showed good adherence, while the Δspa mutant did not adhere (Fig. 5A3 and B3). When the bacteria were resuspended in bovine serum, the wafers were adhered by neither WT *S. aureus* nor Δspa mutant (Fig. 5A4 and B4).

We showed the different adsorption behavior of albumin, γ-globulins and serum on EG_3OMe and PEG2k coatings and the effect on bacterial adhesion. Although, albumin showed little adsorption to the surfaces, it still caused a decrease of *S. aureus* adhesion. γ-globulins strongly adsorbed to the surfaces and promoted *S. aureus* adhesion. But most important was the observation that pre-incubation of *S. aureus* with γ-globulins or serum significantly decreased the adherence to coatings that came in contact with γ-globulins or serum - that is the natural situation of implanted material.

Pretreatment of Pseudomonas aeruginosa with serum significantly decreased its adherence to the coatings

We also carried out similar adherence studies with GFP expressing *Pseudomonas aeruginosa* and obtained similar results as with *S. aureus*. *P. aeruginosa* showed massive adherence to uncoated gold (Au) surfaces (Fig. 7A and B); if the cells were however resuspended with bovine serum adherence to uncoated gold (Au) surfaces was decreased (Fig. 7B). Like *S. aureus*, *P. aeruginosa* hardly adhered to EG_3OMe and PEG2k coated surfaces even when resuspended in buffer (Fig. 7B).

Discussion

The aim of this study was to systematically analyze the adherence behavior of gold (Au) as well as EG_3OMe or PEG2k coated Au surfaces to plasma proteins and pathogenic bacteria

such as *S. aureus*, which plays a crucial role in implant-associated infections. The coating altered the physical appearance of the surface. The sputtered Au surfaces on silicon wafers had a rough and grain-like morphology, which became smoother and flattened by EG_3OMe and even more smooth by PEG2k coating (Fig. 1).

The EG_3OMe and PEG2k coated surfaces were inert to adsorption of the blood proteins lysozyme, fibrinogen and albumin (BSA) if applied at low concentration (1 mg/ml) (Fig. 2) and they were inert to adherence of *S. aureus* (Fig. 4). BSA did not adsorb to the coated surfaces even when applied at very high concentrations (100 mg/ml), which is in line with the literature (Holmlin et al., 2001; Lokanathan et al., 2011; Prime and Whitesides, 1993). However, γ-globulin adsorbed to the EG_3OMe and PEG2k coatings if applied at concentrations higher than 10 mg/ml (Fig. 3); its adsorption could be, however, significantly suppressed if it was mixed with BSA in a ratio of 1:3 as it also occurs in serum. As BSA itself did not adsorb to the EG_3OMe and PEG2k coatings we assume that BSA is neutralizing γ-globulin's binding domains already in solution. Indeed, it has been shown recently that serum albumin has a moderate attraction to IgGs, which may forestall undesirable protein condensation in antibody solutions (Wang et al., 2011). We also found that PEG2k coatings adsorbed four times less γ-globulins than the EG_3OMe coatings. One reason could be that the long polymer chains of PEG2k cover the coating Au substrate more efficiently then EG_3OMe, with the effect that steric repulsion prevents γ-globulin adsorption more efficiently (Jeon and Andrade, 1991). On the other hand, adherence of whole serum was five times higher with the PEG2k coated surface than with the EG_3OMe coated one (Fig. 3D). A possible reason for this observation might be that PEG2k has a much longer thread-like structure than EG_3OMe and revealed a lower surface coverage than the EG_3OMe coating. We assume that small proteins (other than γ-globulins) can more easily penetrate the PEG2k polymer brush thus enhancing the adsorption on PEG2k coatings (Benesch et al., 2001).

The question is why BSA and lysozyme do not bind to the coatings, even at very high concentrations, while γ-globulins do. Serum albumin is the most abundant plasma protein in mammals with an extraordinary ligand binding capacity; these proteins are relatively large (66 kDa) and negatively charged (Majorek et al., 2012). Lysozymes also have a positive net charge that is thought to play an important role in guiding lysozyme to the negatively charged surface of bacteria. The majority of γ-globulins are immunoglobulins (150 kDa), which are also positively charged. Apparently, the charge of the proteins does not play a crucial role in binding to the coatings, as the positive charged lysozyme and γ-globulins show opposing binding effect. Therefore, we assume that specific binding domains in γ-globulins interact

with the PEG2k and EG_3OMe coatings. It would be interesting to know whether it is the Fc or the Fab part of IgG that mediates binding to PEG2k and EG_3OMe.

The pretreatment of EG_3OMe and PEG2k coatings with γ-globulins or bovine serum caused massive adhesion of *S. aureus* (Fig. 4A). This is not so surprising as it is well-known that *S. aureus* is distinguished by its high immunoglobulin-binding activity via its cell-wall bound protein A (Spa) (Löfdahl et al., 1983; Uhlen et al., 1984). The 'second immunoglobulin-binding protein' (Sbi) is secreted and it contributes to complement evasion (Burman et al., 2008; Zhang et al., 1998); but it should not play major role in adherence of cells to the coatings(Burman et al., 2008; Zhang et al., 1998). In this experiment *S. aureus* cells were resuspended in buffer (PBS). However, if we resuspended *S. aureus* cells with bovine or human serum the adherence to the γ-globulins or serum treated coatings was significantly decreased (Fig. 4B). Besides, *S. aureus* Δspa was not detectable on any PEG surfaces, even on the γ-globulins pre-incubated surfaces (Fig. 5). This decreased adherence is due to the saturation of Spa by IgGs present in the serum. Based on this observation implant-associated infections might be minimized by allowing bacterial pathogens to become saturated with the patient's serum.

The function of serum in preventing bacterial adhesion is not restricted to *S. aureus*, we also see this effect with *P. aeruginosa.* One possible explanation could be that the abundant albumin in the serum neutralizes γ-globulins *via* non-specific attraction as mentioned above (Wang et al., 2011), thereby decreasing the bacterial adhesion. With respect to Gram-negative bacteria, like *P. aeruginosa*, one must also consider the complement factors present in serum that might play a role in decreasing bacterial adhesion. Since the composition of serum is complex, many factors might play a role. For instance, it has been reported that apo-transferrin prevented bacterial adhesion to the Tecoflex polyurethane surfaces (Elofsson et al., 1997). Kuroda et al. observed that serum globulins could reduce the adhesion of *Prevotella nigrescens* to hydroxyapatite (Kuroda et al., 2003). Future investigations should focus on identifying more inhibitory components of serum.

On the other hand, pre-incubation of the surfaces with albumin did not promote *S. aureus* adhesion, even when the cells were resuspended with serum. It has been known for a while that surface coatings with albumin have an inhibitory effect on bacterial adhesion. It has been reported that albumin adsorbs to the interface and creates a thin film that prevents bacterial adhesion (Ardehali et al., 2003; Katsikogianni and Missirlis, 2004; Ribeiro et al., 2012). However, the role of albumin is not fully understood. In our experiments, the 'protective' role of albumin on bacterial adhesion can be simply explained by the fact that it is

not adsorbed on the EG_3OMe and PEG2k surfaces and has therefore no further influence on bacterial adhesion. It would be interesting to test in future experiments whether albumin in the solution will inhibit bacterial adhesion onto a surface, especially when the surfaces are pre-incubated with γ-globulins.

In conclusion, our study contributes to a better understanding about the functions of the major serum components on protein adsorption and *S. aureus* adhesion on EG_3OMe and PEG2k coated surfaces. In particular, we found that serum can significantly inhibit the bacteria adhesion on different surfaces, which might have some therapeutic implications in minimizing implant-associated infections.

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Figure legends

Figure 1. AFM height images (1μm x 1μm) of gold surface alone (A), gold surface coated with EG_3OMe (B) or PEG2k (C, D). The images show the surface morphology. The mean squared surface roughness was deduced from the AFM images using software "Gwyddion". By coating the gold surface with EG_3OMe and PEG2k the mean-squared roughness (MSR) decreased more and more, becoming smoother. The yellow to brown color scales beside the images indicate the vertical scale.

Figure 2. Structure and determination of protein adherence to EG_3OMe (A) and PEG2k (B) coatings by PMIRRAS spectral analysis. The peaks seen in the range between 1500 – 900 nm were specific for the coated material EG_3OMe and PEG2k (black spectrum); arrows indicate the main peaks. After incubation with selected blood proteins (1 mg/ml) spectral analysis with potentially adhered proteins was carried out again: lysozyme (green-), fibrinogen (red-) and BSA (blue spectrum). The boxed square indicates the amide I region 1600-1700 cm^{-1}, which is characteristic for protein absorption; there is little adherence of these proteins to EG_3OMe or PEG2k coated gold (Au) surface.

Figure 3. γ-globulin binding to EG_3OMe and PEG2k coated surfaces. (A) PMIRRAS spectra of EG3OMe coated surfaces incubated with different concentrations of γ-globulins. (B) Serum protein adherence was determined by amide I absorbance peak intensity from PMIRRAS spectra: BSA (purple line), γ-globulins (blue line), mixtures of BSA and γ-globulins with a concentration ratio of 3:1 (red line) and lysozyme (Lyz, green line). The concentrations used were 1, 10, 20, 50 and 100 mg/ml. (C) Analysis of serum proteins eluted from EG_3OMe coated surface by SDS-PAGE and Coomassie protein staining. (D) Graphical representation of the adherence capacity of BSA (bovine serum albumin), γ-globulins, γ-globulins together with BSA and bovine serum on EG_3OMe and PEG2k surfaces.

Figure 4. Effect of *S. aureus* resuspended in PBS buffer (A) or serum (B) on adherence to EG_3OMe and PEG2k coated surfaces. Fluorescence microscopy images of *S. aureus* (pC-tuf-ppmch) adhered on uncoated Au surface (1), EG_3OMe coated surface (2), PEG2k coated surface (3) and EG_3OMe coated surfaces pre-incubated with 20 mg/ml BSA (4), 50 mg/ml γ-globulins (5) or 100% bovine serum (6).

Figure 5. Comparison of *S. aureus* WT (A) and protein A mutant (B) on adherence. Fluorescence microscope images of *S. aureus* adhesion on uncoated gold (Au) surfaces (1), PEG5k coated (2) and PEG5k coated surfaces pre-incubated with 50 mg/ml γ-globulins (3 and 4). Wafers were incubated with *S. aureus* SA113 WT (pC-tuf-gfp) or SA113Δspa (pCX-pp-sfgfp); cells were either resuspended in PBS (buffer) or bovine serum (BS).

Figure 6. Quantitative analysis of *S. aureus* (pC-tuf-ppmch) adhesion under different conditions. The coverage of red fluorescent *S. aureus* was calculated by software 'ImageJ', using a size of 1 μm^2 as an input parameter. The analyzed square seen in Fig. 3 was approx. 200 μm x 120 μm). The name for each block indicates the experimental conditions as 'surface pre-incubation *vs.* bacteria suspension'; for example: 'BSA+serum' means that the surfaces were pre-incubated in BSA and the bacteria were resuspended in serum. Blue bars, uncoated Au surfaces; red bars, EG_3OMe coated surfaces; green bars, PEG2k coated surfaces.

Figure 7. Adherence comparison between *S. aureus* and *P. aeruginosa*. (A) Fluorescence images on uncoated gold (Au) surfaces after incubation with *S. aureus* or *P. aeruginosa* in PBS buffer. (B) Comparison of the cell counts on uncoated gold (Au) surfaces, EG_3OMe

coated and PEG2k coated surfaces for *S. aureus* and *P. aeruginosa* resuspended in PBS buffer or bovine serum.

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657

Figure 1

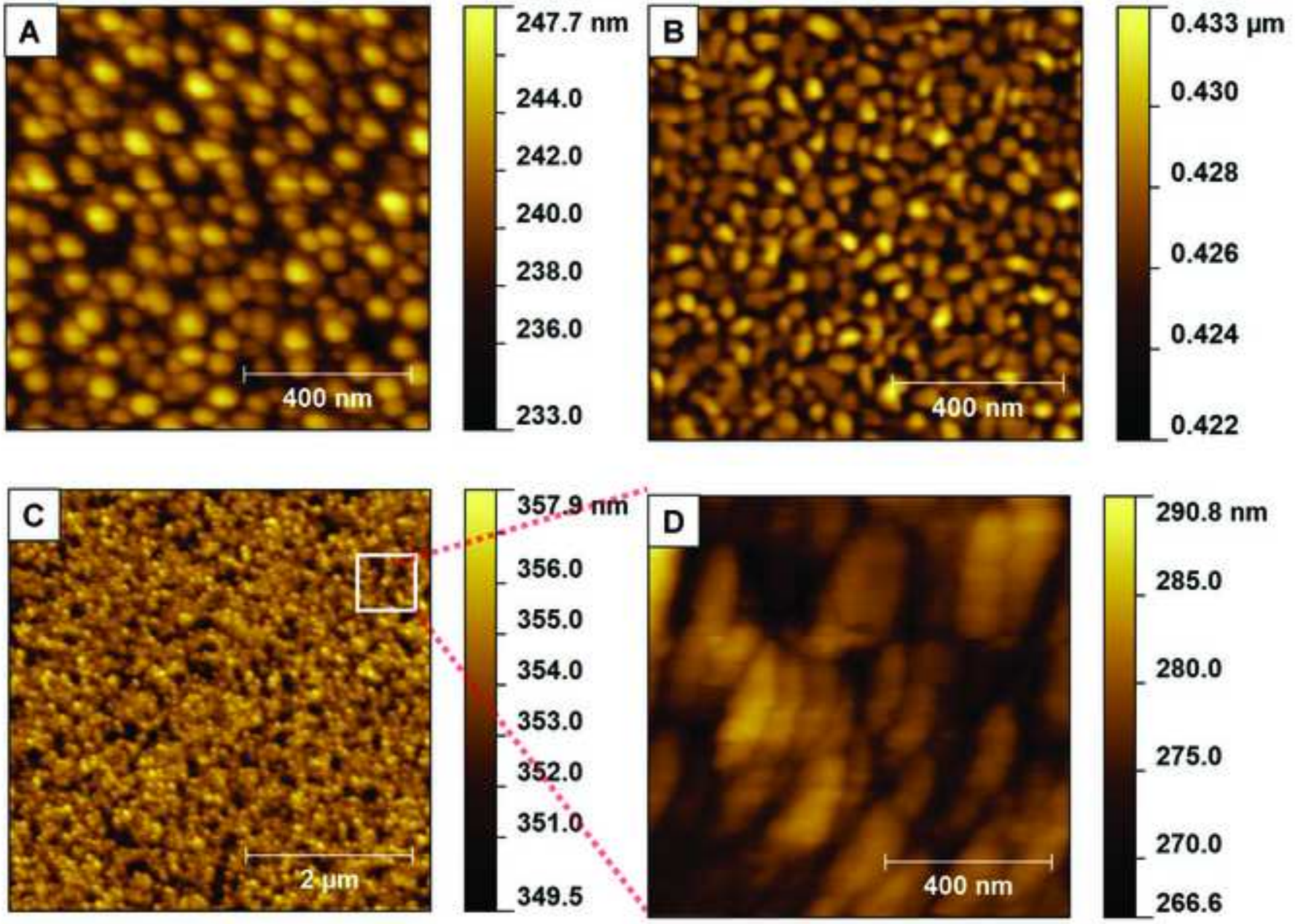

Fig. 1

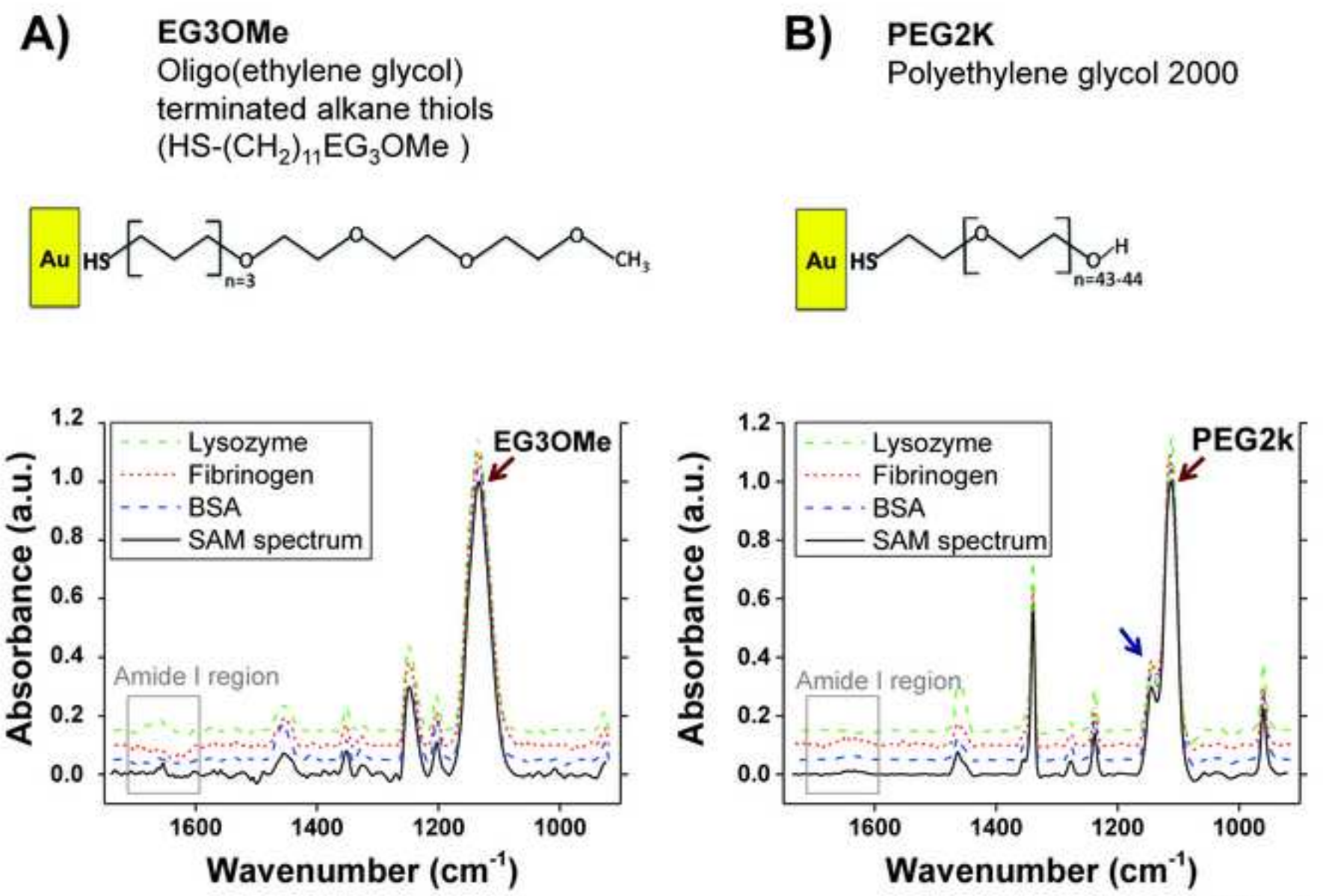
A)
EG3OMe
Oligo(ethylene glycol)
terminated alkane thiols
(HS-(CH2)11EG3OMe)
Au
HS
n=3
CH3
B)
PEG2K
Polyethylene glycol 2000
Au
HS
H
n=43-44
Absorbance (a.u.)
Lysozyme
Fibrinogen
BSA
SAM spectrum
EG3OMe
Amide I region
Wavenumber (cm-1)
PEG2k

Fig. 2

Figure 3

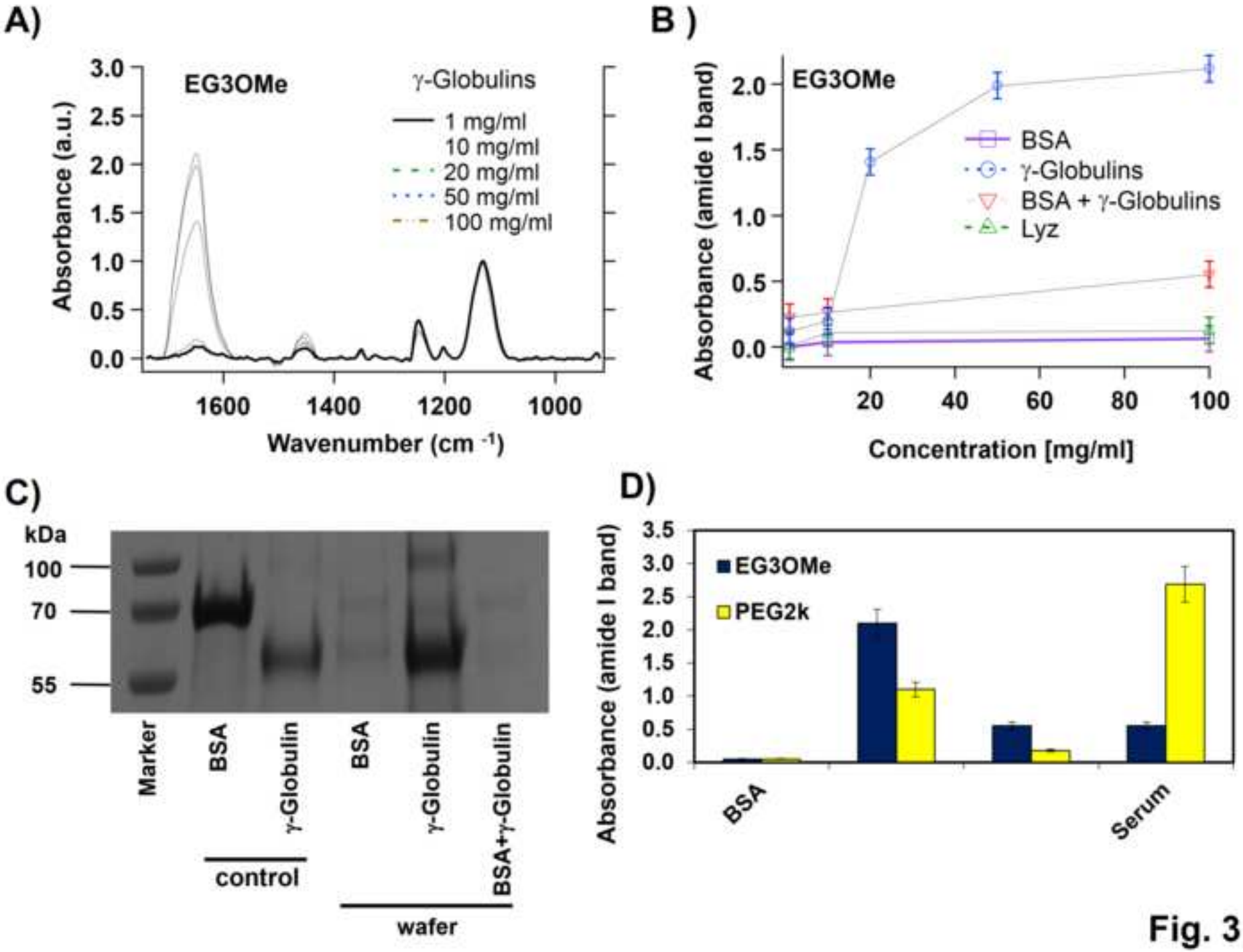

Fig. 3

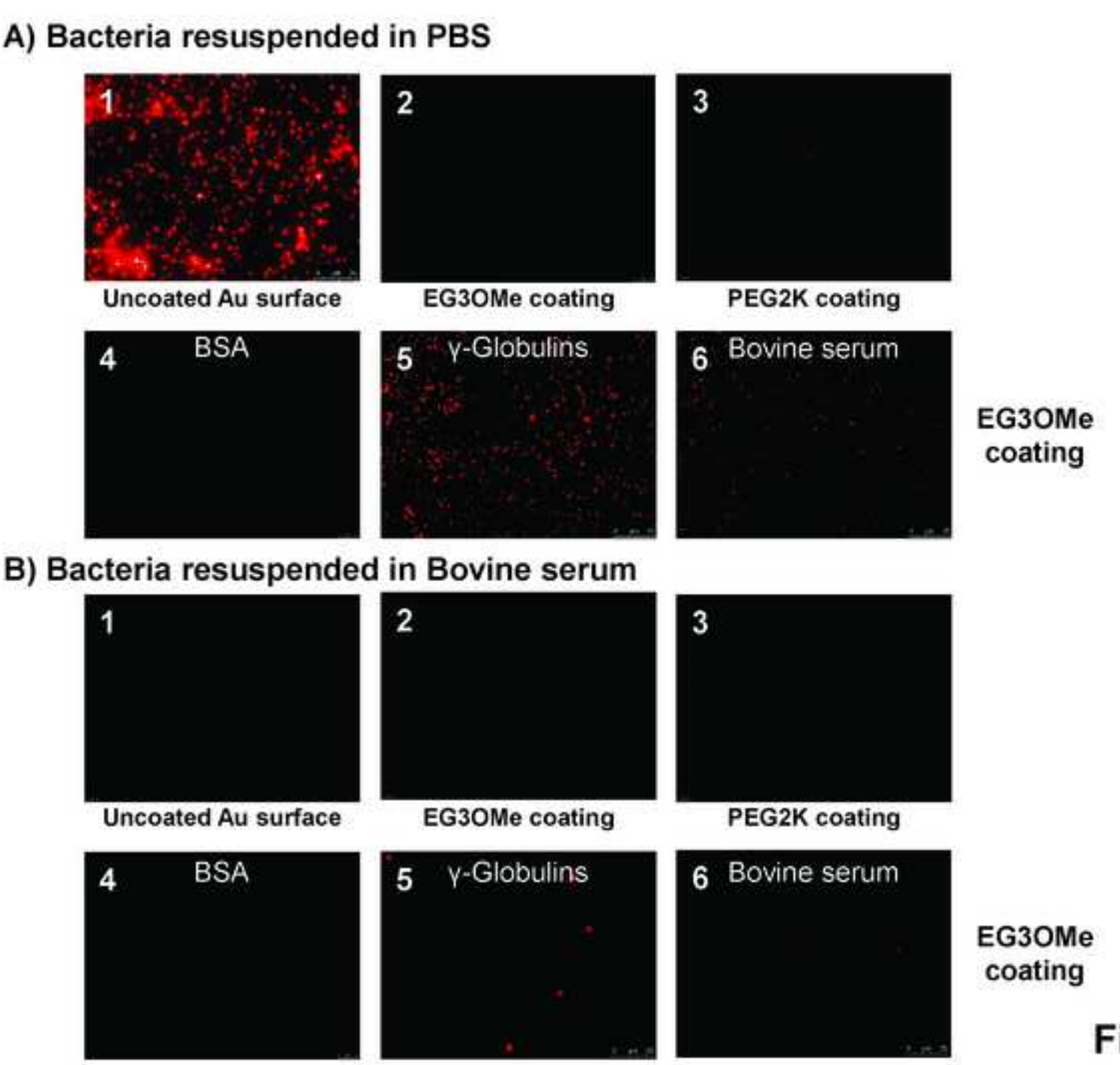
A) Bacteria resuspended in PBS
1
2
3
Uncoated Au surface
EG3OMe coating
PEG2K coating
4 BSA
5 γ-Globulins
6 Bovine serum
EG3OMe coating
B) Bacteria resuspended in Bovine serum
1
2
3
Uncoated Au surface
EG3OMe coating
PEG2K coating
4 BSA
5 γ-Globulins
6 Bovine serum
EG3OMe coating

Fig. 4

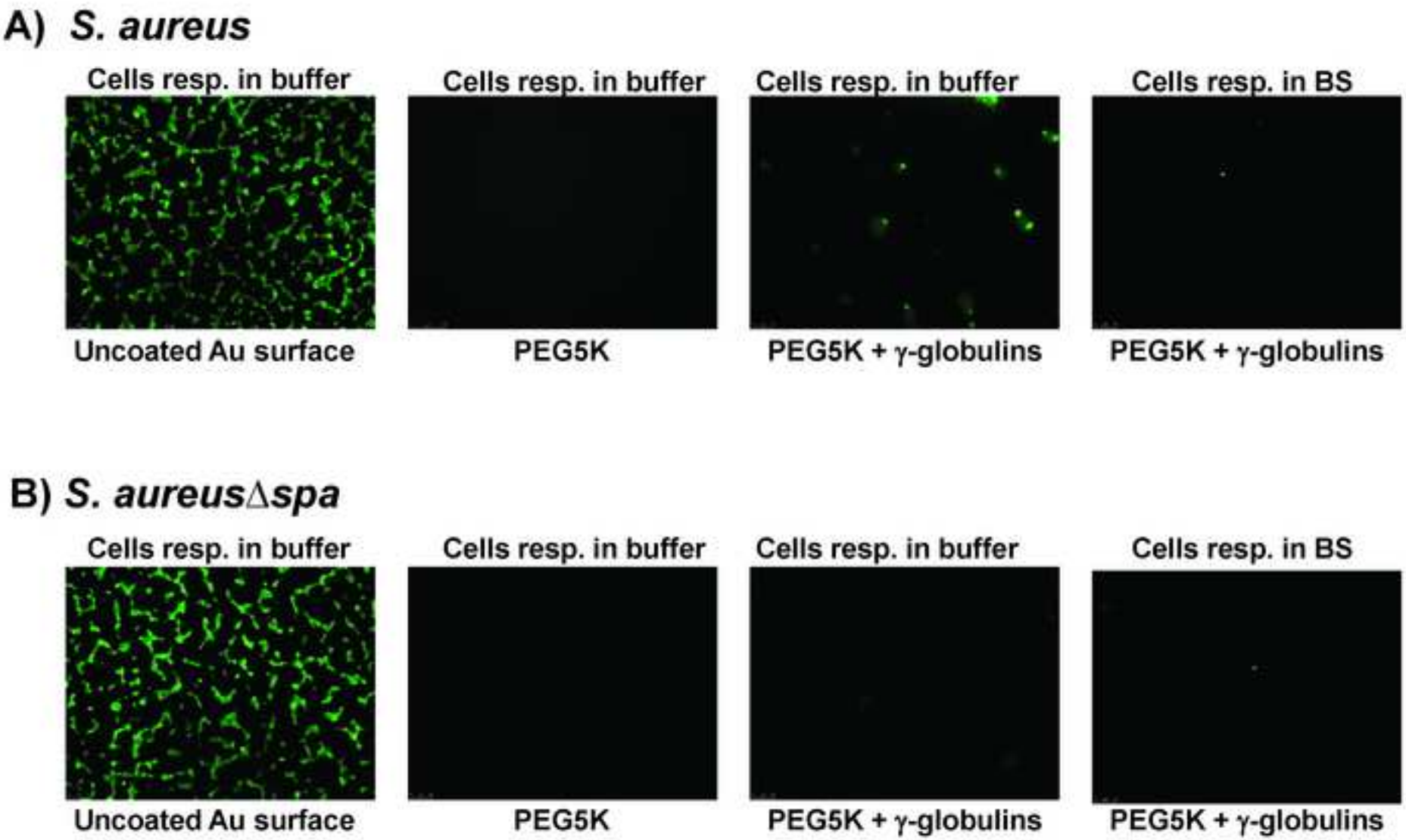
A) S. aureus
Cells resp. in buffer
Cells resp. in buffer
Cells resp. in buffer
Cells resp. in BS
Uncoated Au surface
PEG5K
PEG5K + γ-globulins
PEG5K + γ-globulins
B) S. aureusΔspa
Cells resp. in buffer
Cells resp. in buffer
Cells resp. in buffer
Cells resp. in BS
Uncoated Au surface
PEG5K
PEG5K + γ-globulins
PEG5K + γ-globulins

Fig. 5

Figure 6

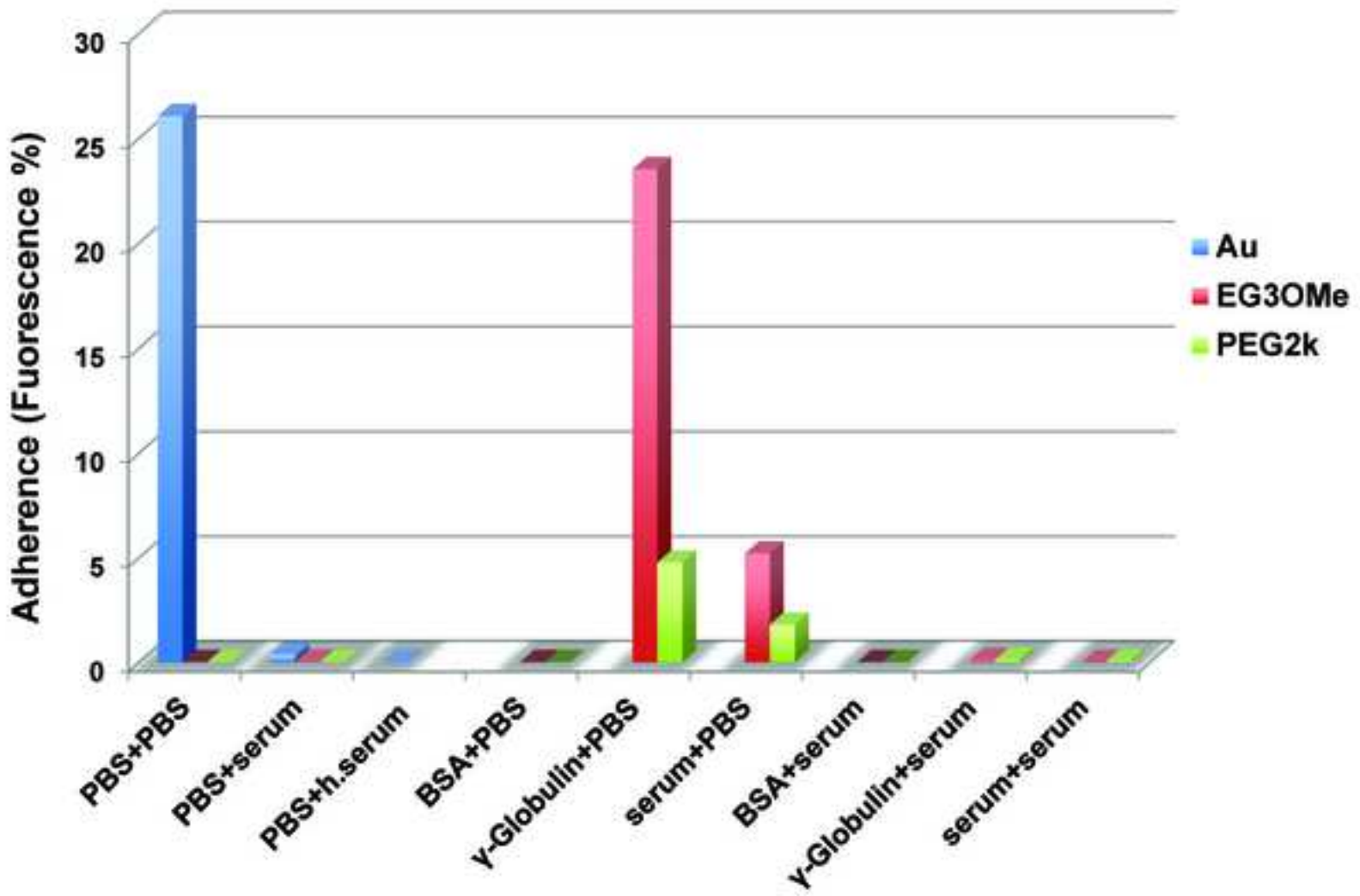

Fig. 6

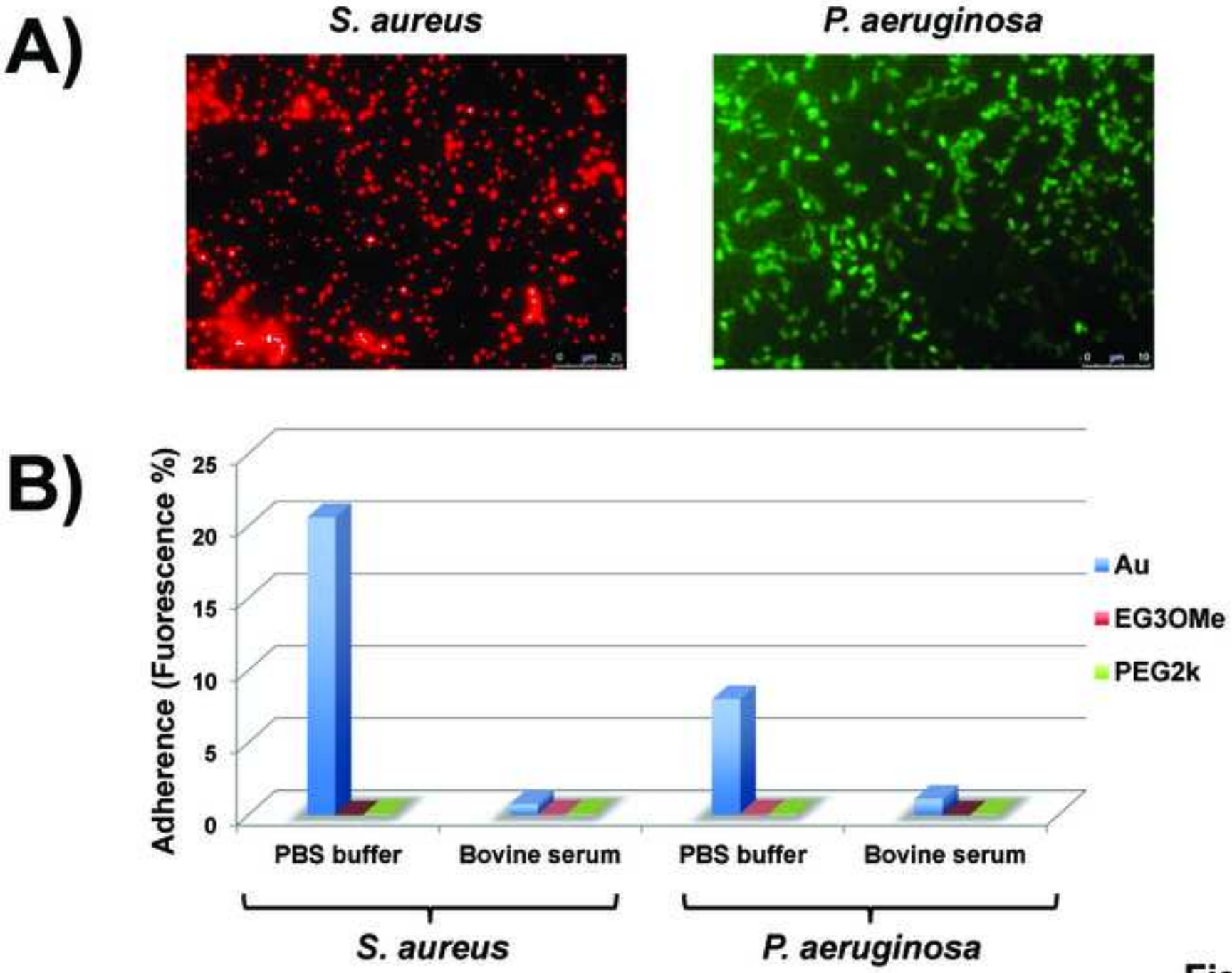
A)
S. aureus
P. aeruginosa
B)
Adherence (Fuorescence %)
25
20
15
10
5
0
PBS buffer
Bovine serum
PBS buffer
Bovine serum
S. aureus
P. aeruginosa
Au
EG3OMe
PEG2k

Fig. 7